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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. LVII SEPTEMBER, 1929 NO. 3

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PUBLISHED MONTHLY BY THE

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, INC.

LANCASTER, PA.

AGENT FOR GREAT BRITAIN

WHELDON & WESLEY, LIMITED

2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2

Single Numbers, $1.00. Per Volume (6 numbers), $4.50

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894

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Vol. LVII *September, 1929* *No. 3*

BIOLOGICAL BULLETIN

THE SO-CALLED CENTRAL BODIES IN FERTILIZED *ECHINARACHNIUS* EGGS.

II. The Relationship between Central Bodies and Astral Structure as Modified by Various Fixatives.[1]

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Statement of the Problem.

It is generally agreed among cytologists that central bodies (centrosomes, centrioles) are the formative foci about which arises the mitotic mechanism of the animal cell. There is diversity of opinion, however, concerning whether or not they exhibit genetic continuity as individualized structures from cell to cell and from generation to generation. The behavior of central bodies in echinoderm fertilization has been regarded, heretofore, not only as supporting the theory that they are the formative foci of asters, but also as yielding evidence in favor of their genetic continuity from generation to generation.[2]

[1] The material was secured at the Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] A review of the literature concerning central bodies in echinoderm fertilization, together with a brief discussion of terminology, will be found in the first paper of this group (Fry, '29, pp. 101–109). A further discussion will be found in the present study, pp. 143–147.

There is much confusion concerning terminology in the literature because a given term is used by various authors with different meanings. In the present group of papers the term *centriole* applies only to a minute period-like type of central body; the term *centrosome* applies only to a larger, more diffuse type that is often vaguely delimited; the term *central body* is a broad one including both the others. It is unfortunate that in the previous paper (Fry, '29) the inclusive non-committal term *central body* was used most frequently. It is better to avoid this ambiguous term as much as possible, using instead the more specific words *centriole* and *centrosome*. This procedure is carefully followed in the present paper.

In the preceding investigation (Fry, '29) central bodies in *Echinarachnius* eggs were studied at various intervals from fertilization to first cleavage, after fixation with Bouin's fluid. The following conclusion was reached, based upon a detailed analysis of over 900 eggs. *The so-called centrosomes of fertilized Echinarachnius eggs are nothing but coagulation artifacts of ray substance at the astral center, produced by the fixative only during those periods when rays are well formed.* Both sperm-asters and cleavage-amphiasters pass through an early vague-rayed period during which there are no centrosomes; not until the rays become clear do centrosomes appear; when such well formed rays fade the centrosomes disappear. These facts are in harmony with central body phenomena during the history of cytasters in artificially activated eggs (Fry, '28, Chart III.). *The so-called centrioles of fertilized Echinarachnius eggs are nothing but cytoplasmic granules.* They are usually abundant among the astral rays but are generally absent in the mid-region. They occasionally occur there, however, in various positions and in various numbers. In a very limited percentage of cases such granule configurations simulate centrioles.

If the hypothesis is correct that centrosomes of fertilized *Echinarachnius* eggs are actually coagulation artifacts of the focal point of clearly fixed rays, then no matter by what means astral structure is modified, centrosomes should be present in coagulated asters only when rays are clearly fixed, and they should be absent when rays are fixed vaguely or not at all. This relation-

ship should hold good whether ray structure is modified by successive phases in the normal astral cycle, or by different coagulation effects of various fixatives, or by modifications due to environmental factors, or by other causes.

In the previous study of the effect of different mitotic stages upon rays and central bodies the environment is constant, the living eggs being kept in normal sea water at 20° C. The fixative is also constant; Bouin's fluid is used because it is one of the standard reagents that clearly coagulates rays. The variable is the astral structure: different kinds of ray configurations, varying from vague to clear, occur during the successive stages of the astral history, thus giving an opportunity to study the relationship between the presence of centrosomes and the occurrence of clear rays reaching the center.

In the present study the environment is constant as above. The astral stage is also constant, only metaphase asters being selected for study because when properly fixed they show clear rays and typical echinoderm central bodies. The fixative is the variable: different kinds of ray configurations, varying from vague to clear, are produced by a wide variety of coagulating agents, thus giving another opportunity to study the relationship between clear rays and centrosomes.

In this experiment the fertilized eggs were kept in normal sea water at 20° C. They were simultaneously placed in a wide variety of coagulating agents one hour after insemination, the time when metaphase asters are most abundant. A study of such asters with water-immersion objectives shows that they have a similar structure in the living condition: the outer portion has a clearly radiate configuration, although the details are indistinct; the center is hyaline and apparently structureless. Hence the modifications of metaphase astral structure analyzed in the present investigation are due to the differing coagulation effects of various fixatives acting upon metaphase asters that were relatively similar in the living eggs.

If the proposed hypothesis is correct that centrosomes in fertilized *Echinarachnius* eggs are nothing but coagulation artifacts of the focal point of clearly fixed rays and have no existence as individualized bodies in the living condition, then in the present

study they should be present only in the case of those reagents that coagulate the radial configuration of the living metaphase asters in such a manner that the fixed asters have clear rays. They should be absent when rays are fixed vaguely or not at all. Furthermore, if centrosomes are actually coagulation products of the inner ends of rays, they should also show a very great diversity in structure in the various reagents. Such modifications should be far more diverse than those that would be produced by different fixatives acting upon a component such as chromosomes which have an actual existence as formed bodies in the living cell.

The Method of Study.

Fertilized *Echinarachnius* eggs were simultaneously fixed in 128 reagents one hour after insemination. These fixatives comprise the standard ones, together with their components used separately, as well as various unusual combinations of reagents.[3] In the case of each fixative, about ten metaphase figures, chosen at random, were each measured and analyzed with reference to the twenty-two points listed in Table I. Upon the basis of this preliminary examination, the coagulation products of twenty-seven fixatives were chosen for illustration, and in the case of each one, an additional fifteen metaphase figures were similarly analyzed.[4] Those selected for intensive study were chosen to illustrate the following points: (1) to show the effects of representative standard fixatives, *i.e.*, sublimate-acetic (Fig. 3), Carnoy (Fig. 11), Petrunkewitsch (Fig. 12), Burckhardt (Fig. 15), Zenker (Fig. 16), Flemming (Fig. 19), and Bouin (Fig. 27); (2) to show the effects of special fixatives devised to demonstrate Golgi-bodies and chondriosomes, *i.e.*, Kopsch (Fig. 17), Benda (Fig. 18), Flemming (Fig. 19), Hermann (Fig. 20), formol, neutralized (Fig. 21), and Regaud (Fig. 22); (3) to show the ef-

[3] A joint study with Dr. H. H. Darby is now in progress concerning the effect of pH upon the coagulation of asters in many fixatives. Some of the slides of that study were used in the present investigation; in such cases the fixative is at its normal pH.

[4] Appreciation is expressed to Miss Sara Jane Reynolds for her help as research assistant in making the thousands of measurements and observations involved in the preparation of this paper, as well as the others of the series.

ASTER.

Shape and dimensions.
Whether or not differentiated into zones: in region about central body; in outer part.
Stain in contrast to that of the cytoplasm.

ASTRAL RAYS.

Whether clearly or vaguely fixed.
Whether delicate or coarse.
Whether a continuous line or a series of granules.
Whether relatively straight and largely separate from each other, or crooked and anastomosing.

"CENTROSOME."

(*i.e.*, large diffuse portion of central body.)

Shape and dimensions.
Stain in contrast to that of ray area.
Physical structure, *i.e.*, whether entirely homogeneous, or coarsely granular, or mulberry-like, or reticular, or vacular, etc.
Whether or not traversed by rays.
Whether or not clearly delimited from the ray area.

"CENTRIOLE."

(*i.e.*, small, period-like granule, or granules).

Number and size.
Location within the "centrosome."

CYTOPLASMIC GRANULES.

Abundance among rays: in outer part of aster; in inner part.
Comparison with "centrioles."

SPINDLE.

Length and width.
Stain in contrast to that of aster.
Structure of spindle fibers in contrast to that of astral rays.

CHROMOSOMES.

Size.
Exact location and grouping.

TABLE I.

Points with reference to which cleavage (metaphase) figures are measured and analyzed in the case of each fixative.

fects of the various components of the above fixatives when used separately, *i.e.*, corrosive sublimate (Fig. 1), chloroform (Fig. 7), absolute alcohol (Fig. 9), potassium dichromate (Fig. 13), chromic acid (Fig. 14), osmic acid (Fig. 17), formol, neutralized (Fig. 21), formol, acid (Fig. 24), acetic acid (Fig. 25), and picric acid (Fig. 26); (4) and finally, to show all the types of coagulation products present in the 128 fixatives originally examined.

In the previous study in which Bouin's fluid was used the metaphase asters fall into two clearly demarked classes. In "early" metaphase asters (Fry, '29, Fig. 21) the chromosomes of the metaphase plate are in a tangled group; the rays are very delicate; there is no centrosome. In "late" metaphase asters (Fry, '29, Fig. 22) the chromosomes of the metaphase plate are aligned in two closely approximated groups that are ready to separate; the rays are coarse; there is a pleuricorpuscular centrosome.[5] In the case of many fixatives of the present study there are no differences between "early" and "late" metaphase stages, but whenever such differences do exist, the "late" metaphase asters were studied.

In many of the fixatives selected for illustration all the metaphase ("late") asters fall into one class with reference to the central body (centrosome). In the remainder there are two or three classes; in such cases that class occurring in largest numbers is illustrated. If more than one type of coagulated metaphase aster occurs in a fixative, this situation is usually caused by various degrees of destaining among different eggs of the same slide, a factor that may produce marked differences in appearance. More rarely the differences are due to the fact that the metaphase asters of different eggs are not coagulated in exactly the same manner; this is apt to occur in those fixatives that coagulate rays very faintly.

The formulae used are those listed in Lee's "The Microtomist's Vade Mecum." Eggs were left in the fixative about two hours. The methods of running up are those of the usual practise as described by Lee. In the case of the Golgi-body fixatives (Kopsch,

[5] The previous illustration of metaphase asters after Bouin's fixation (Fry, '29, Fig. 22) shows the centrosome too dark and too sharply demarked. Such asters are correctly illustrated in the present paper (Fig. 27).

Fig. 17; Benda, Fig. 18; formol, neutralized, Fig. 21; and Regaud, Fig. 22), after fixation the eggs were osmicated at various intervals from one to fourteen days at room temperature; they were immediately run up and embedded without being allowed to stand in the alcohols. Eggs fixed in those reagents used for the demonstration of chondriosomes (Benda, Fig. 18; Flemming, Fig. 19; Hermann, Fig. 20; and Regaud, Fig. 22) were chromated at various intervals from three to ten days. Eggs were sectioned 5 μ thick. They were stained with Heidenhain's haematoxylin. They were studied at 750 $\times$ magnifications.

The figures (pp. 140–141) are arranged in horizontal columns with reference to various fixative groups, such as those using mercuric chloride, alone and in combination (Group I.); those using chloroform, alone and in combination (Group II.); etc. Underneath each figure is given the formula, and the name, if such occurs, by which the fixative is generally known. In addition to the horizontal arrangement with reference to fixatives, there is a vertical grouping with respect to whether or not rays are clearly fixed. The fixatives included in the narrow left hand column fix rays very vaguely or not at all; those in the broad right hand column fix rays clearly, whether they be coarse or delicate.

The figures are 650 $\times$ enlarged. The diameter of the coagulated aster in Fig. 3, for example, is 28 μ. In each figure, every dimension and the physical appearance of each part is an average of all the observations made with reference to the points listed in Table I., in all the individuals studied, belonging to that class of ("late") metaphase asters occurring in largest numbers, in that fixative. The chromosomes, however, are an exception to this, as in each case they were drawn from a single cell and were studied only superficially.

The method of study used in the present work is the same as that previously described (Fry, '28, pp. 387–392; '29, pp. 109–113). At each stage, using a given fixative, there is selected at random a sufficiently large number of cells to constitute an adequate random sample. They are all measured and analyzed in the greatest detail possible, and are classified in their various groups; the percentage of each class is noted. The interrelationships of the varying factors are observed in each group. This

method is repeated in a number of diverse fixatives. Any conclusion is based upon a consideration of many classes of coagulation products, knowing the percentage of each, and the manner in which the different variable factors are interrelated in the various classes.

The Effects of Various Fixatives upon the Relationship between Clearly Fixed Rays and Central Bodies in Metaphase Asters of Fertilized *Echinarachnius* Eggs.

When metaphase asters of the first cleavage figure in *Echinarachnius* eggs are coagulated by a variety of fixatives, centrosomes are present only in the case of those reagents that fix rays clearly whether they be coarse or delicate; they are absent when rays are fixed vaguely or not at all (Figs. 1–27). This result is in harmony with that obtained in the study of the effect of fixatives upon cytasters in artificially activated eggs (Fry, '28, Chart I.).

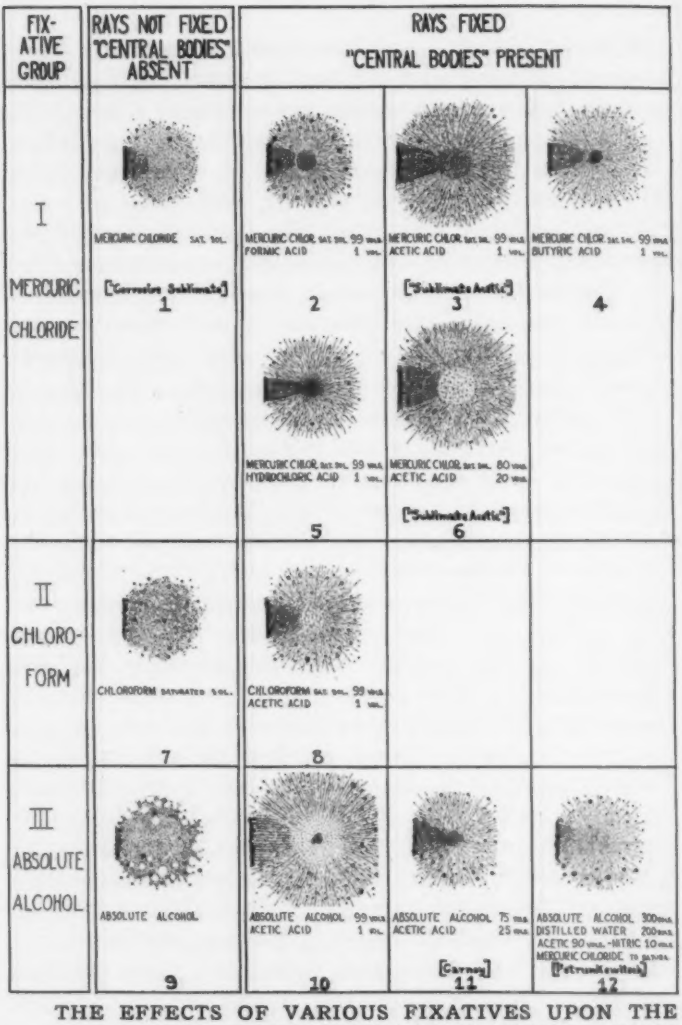
The clear fixation of rays does not necessarily involve the presence of a definitely demarked centrosome (Figs. 12 and 23), although such is usually the case. The same situation holds true in cytasters (Fry, '28, Chart I., Type D1). Although centrosomes may occasionally be absent when rays are clearly fixed, they are never present unless rays are clearly fixed, and they are always absent when rays are fixed vaguely or not at all.

The different reagents produce a wide and significant diversity in the structure of centrosomes. They may be small and clearly delimited, whether granular (Fig. 4), or vacular (Fig. 10). They may be large and clearly delimited, whether granular (Figs. 2 and 26), or pleuricorpuscular (Fig. 27, etc.), or vacular (Fig. 6, etc.). They may be vaguely delimited, whether granular (Fig. 23, etc.), or vacular (Fig. 8, etc.). Various types of zoning occur about them, and there are other detailed modifications. If centrosomes have an actual existence in the living cell as individualized definitely-formed structures, it would be expected that most of their coagulation products would be somewhat similar to each other, as is the case with respect to the chromosomes. The extensive variability in centrosome structure caused by the fixatives, coupled

with the fact that they occur only when rays are clearly coagulated, is further indication that they are nothing but coagulation products of the focal point of the rays.

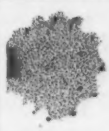
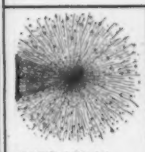
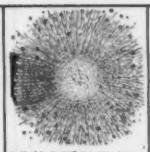
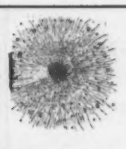
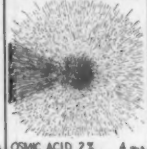
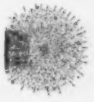
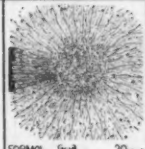
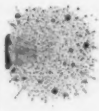
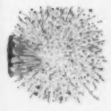
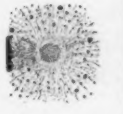
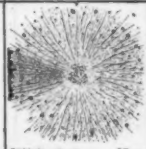
No correlation can be observed between the various types of ray configurations and the different classes of centrosomes. They may be definitely delimited and granular, whether rays are coarse (Fig. 16), or delicate (Fig. 4); or they may be vaguely delimited and vacular, whether rays are coarse (Fig. 15) or delicate (Fig. 8). The variations in centrosome structure are probably explained by the diverse chemical effects of the different reagents acting upon the material at the astral center, independently of whether or not the rays have a coarse or a delicate fixation.

Not only does the fixative modify the centrosome structure of metaphase asters, but it also modifies the exact time when centrosomes appear and disappear. Large numbers of asters in all stages from early prophase to late telophase were studied in the following reagents: corrosive sublimate, sublimate-acetic, absolute alcohol, absolute alcohol and acetic acid, Burkhardt's reagent, Benda's reagent, Flemming's fluid, Bouin's fixative, and a picro-acetic mixture. The results of this phase of the work are not illustrated. In those reagents where rays are clearly fixed and hence centrosomes occur, the exact time of their appearance and disappearance differs in the various fixatives. In some cases centrosomes arise during prophase, in others not until metaphase. The exact time when the enlarged central body (centrosphere) of the later stages disappears also shows variability in the various reagents. This result is in harmony with the behavior of central bodies in cytasters (Fry, '28, Chart III.), where the time of their appearance and disappearance shows considerable variability in different fluids. Such differences in the time schedule are explained by the fact that a given reagent may cause the clear coagulation of rays during prophase, whereas another fluid does not accomplish this until metaphase. Hence the central bodies make their appearance at different stages, depending upon the fixative used. This is further evidence that central bodies are nothing but coagulation products of the focal point of clearly fixed rays, since they appear and disappear simultaneously with the appearance and disappearance of such rays.



THE EFFECTS OF VARIOUS FIXATIVES UPON THE AND CENTROSOMES IN METAPHASE ASTERS

Asters are in the metaphase ("late") stage. They were similar in structure in the living eggs. The present differences are due to the various coagulation effects of different fixatives. In each figure each dimension and the physical structure of each part is an average of the observations made in a number of asters, studied with reference to the points listed in Table I., p. 135. The chromosomes, however, were studied only superficially. The figures are 650 × enlarged. The diameter of the aster in Fig. 3, for example, is 28 μ. Asters are grouped horizontally with reference to classes

FIXATIVE GROUP	RAYS NOT FIXED "CENTRAL BODIES" ABSENT	RAYS FIXED "CENTRAL BODIES" PRESENT		
IV POTASSIUM DICHROMATE / CHROMIC ACID	 POTASSIUM DICHROMATE 1% 13	 CHROMIC ACID 1% 14	 POTASS. DICHROM. 5% 30 ccs. CHROMIC ACID 1% 60 ccs. ACETIC ACID 5 ccs. [Burckhardt] 15	 POTASSIUM DICHROM. 2.5 gms. SODIUM SULPHATE 1 gm. MERCURIC CHLORIDE 5 gms. ACETIC 5 c.c.-WATER 100 c.c. [Zenker] 16
V OSMIC ACID	OSMIC ACID 2% [Kopsch] 17	OSMIC ACID 2% 4 ccs. CHROMIC ACID 1% 15 ccs. DISTILLED WATER 19 ccs. [Benda] ["Flemming without acetic"] 18	OSMIC ACID 2% 4 ccs. CHROMIC ACID 1% 15 ccs. ACETIC ACID 1 cc. DISTILLED WATER 20 ccs. [Weak Flemming] 19	 OSMIC ACID 2% 4 ccs. PLATINIC CHLORIDE 1% 15 ccs. ACETIC ACID 1 cc. [Hermann] 20
VI FORMOL [NEUTRAL]	FORMOL [brom. und. phos.] 10% 21	 FORMOL [acid] 20 ccs. POTASS. DICHROM. 3% 80 ccs. [Regaud] 22	 FORMOL [acid] 20 ccs. POTASS. DICHROM. 3% 80 ccs. ACETIC ACID 1 cc. [Regaud] 23	
VII FORMOL [ACID] / ACETIC ACID / PICRIC ACID	 FORMOL [acid] 10% 24	 ACETIC ACID 1% 25	 PICRIC ACID sat. sol. 26	 FORMOL [acid] 25 ccs. PICRIC ACID sat. sol. 75 ccs. ACETIC ACID 5 ccs. [Bouin] 27

RELATIONSHIP BETWEEN CLEARLY FIXED RAYS OF FERTILIZED *ECHINARACHNIUS* EGGS

of fixatives: they are grouped vertically with reference to whether or not rays are clearly fixed. Central bodies (centrosomes) are present only in those fixatives that clearly coagulate rays; they are absent when rays are not clearly fixed, even though in such cases the spindle fibers may be coagulated. The very wide diversity in centrosome structure is significant. These facts indicate that the so-called central bodies (centrosomes) of *Echinarachnius* are nothing but coagulation artifacts of the focal point of clearly fixed rays.

It has been suggested that central bodies may have the same chemical composition as that of rays, hence a fixative that fails to clearly coagulate rays will therefore probably fail to show central bodies. The answer to this is as follows. In the previous study (Fry, '29), during telophase stages (Figs. 25 and 26), rays are fixed very clearly in the outer part of the aster whereas they are vague or absent in the central area. These asters have no centrosomes, although the fixative is capable of clearly coagulating rays in the peripheral part of the asters, and also shows centrosomes in the earlier stages where clear rays reach the center. Similarly, in the study of cytasters (Fry, '28) there is a large number of asters (Types C1 and C2) without central bodies, where rays are clearly fixed peripherally but are absent centrally. If central bodies have the same chemical composition as the rays they should be demonstrated in such cases, since the fixative is capable of clearly fixing rays in the outer part of the asters. Another source of evidence, in this connection, is found in three instances (Figs. 1, 17 and 24) in which the spindle fibers are quite clearly coagulated, whereas the rays are practically unfixed and centrosomes are absent. Since the fixative is able to coagulate the spindle fibers in these cases, and since it coagulates the rays very vaguely in two of them (Figs. 1 and 24), it should also show centrosomes assuming that the latter have the same chemical composition as rays and spindle fibers. Such anastral figures, produced by the fixative, in which the centrosome is absent, indicate that centrosomes are present only when clearly fixed astral rays converge from all sides; the clear fixation of the spindle fibers without such fixation of rays does not produce centrosomes.

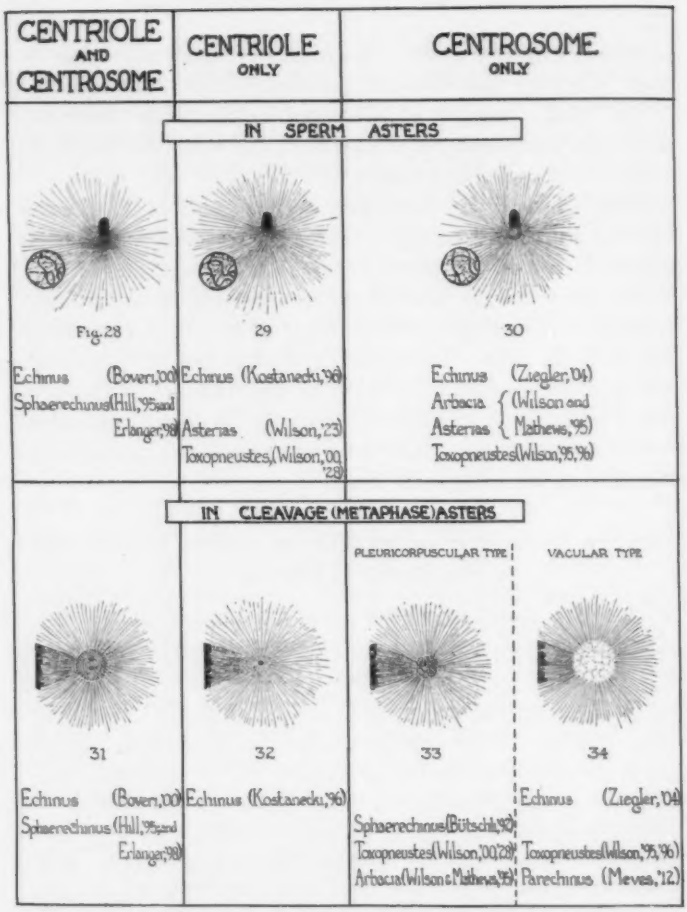
It has also been suggested that only those fixatives that penetrate rapidly may give an accurate coagulation picture of the living condition, and that they alone may be capable of clearly fixing rays and of showing the central body; whereas, fixatives that penetrate slowly may cause a partial break down of the cell structures so that rays are unfixed and the central body is not shown. The answer to this suggestion is as follows. Aside from the fact that data are unavailable concerning which reagents penetrate rapidly or slowly, it is not necessarily a valid assumption that a rapidly

penetrating fixative gives a coagulation product more like the living condition than one that penetrates slowly. The concepts "slow" and "rapid" when applied to molecular dimensions within the protoplasm are of doubtful value with reference to the validity of coagulation products.

Thus, whether the coagulated ray structure of fertilization asters in *Echinarachnius* eggs is modified by various fixatives (the subject of the present paper), or by various stages in the astral history (Fry, '29), or whether similar modifications are produced in cytasters of artificially activated eggs (Fry, '28), the same conclusion holds good. The so-called centrosomes of *Echinarachnius* eggs are coagulation artifacts of the substance at the astral center, produced by fixation only when rays are clearly coagulated and reach the mid-point; they have no existence as individualized structures in the living condition.

The Status of Echinoderm Central Bodies in the Light of the Present Investigation.

The chart presenting Figures 28 to 34 (p. 144) constitutes a résumé of former studies of central bodies in echinoderm fertilization (cf. Fry, '29, pp. 105–109; 120–121). Both in sperm-asters and in cleavage-asters earlier investigators describe the same three classes of central bodies: (1) a type composed of a minute period-like granule, the centriole, surrounded by a larger diffuse structure, the centrosome; (2) a type composed of a minute centriole only, without a centrosome; (3) a type composed only of the larger structure, the centrosome, without a centriole. In this classification the terms "centriole" and "centrosome" are used in the same manner as previously discussed (p. 132). Underneath each figure is given a list of the species which have been described as possessing that type of central body, together with the investigators reporting the observations. No attempt has been made to make the lists complete but they include the major papers in the field. Although the drawings of the various classes are in each case a diagrammatic presentation of several original figures made by different workers to illustrate the situation in various species, they are, nevertheless, accurate delineations of the major details of the originals.



TYPES OF CENTRAL BODIES PREVIOUSLY DESCRIBED IN FERTILIZED ECHINODERM EGGS

The drawings diagrammatically illustrate central body structures previously described in echinoderm fertilization. There are three classes: (1) those composed of a centriole and a centrosome; (2) those composed of a centriole only; (3) those composed of a centrosome only. The upper figures illustrate the situation in sperm-asters; the lower ones show cleavage (metaphase) asters. Underneath each illustration is a list of the species having that class of central bodies, together with the investigators reporting the observations. The present study indicates that previously described echinoderm "centrosomes" are actually coagulation artifacts of the focal point of clearly fixed rays, and that "centrioles" are actually cytoplasmic granules that occasionally occur at the mid-point of the asters.

It will be observed that a minute period-like type of central body, is described by many of the investigators. This fact supports the assumption that in many cases the central body in echinoderm fertilization is a typical centriole similar to the structure supposed to occur generally in animal cells. On the other hand, it is apparent that the previous studies show a considerable diversity in central body structure. In *Echinus*, for example, in both sperm- and cleavage-asters, all three types of central bodies are reported by various workers. Similarly, the phenomena in *Asterias*, *Toxopneustes* and *Sphaerechinus* are variously described. The causes of these discrepancies are explained by the present study.

The central body type composed of both centriole and centrosome (Fig. 31) is described most fully by Boveri ('00) in *Echinus*. He used a picro-acetic fixation composed of one part glacial acetic acid, and ninety nine parts of a saturated solution of picric acid diluted with two volumes of water. At Boveri's suggestion, Coe investigated the effects of various fixatives upon sea urchin eggs. Boveri states: "the sections prove that none of the mediums used surpass the picro-acetic mixture; the best of them does not even approach it" ('00, p. 30). The central body structure he describes in metaphase asters of *Echinus* eggs produced by his picro-acetic fixative (Fig. 31; cf. Fry, '29, Figs. 7–10), is very similar to that occurring in *Echinarachnius* eggs when similarly fixed. In both species there is a large, homogeneously granular centrosome. Furthermore, if the situation in *Echinus* is similar to that in *Echinarachnius*, the supposed centrioles illustrated by Boveri, are actually cytoplasmic granules that happen to occur at the astral center. In the present investigation the cytoplasmic granules were carefully studied, and there is wide variety in this respect. In the case of some fixatives such granules are absent (Fig. 18, etc.); in others they are small, whether few (Fig. 23, etc.), or numerous (Fig. 14, etc.); in others they are large, whether few (Fig. 24, etc.), or numerous (Fig. 26, etc.). In the case of picro-acetic fixation the granules are very numerous. In all fixatives they tend to be excluded from the central portion of asters, and are abundant in the outer parts; they do occur sporadically, however, in the central areas. The more abundant they are in the cytoplasm generally, the more apt are they to be found at the astral

center, hence fixation with picro-acetic favors their occasional occurrence near the mid-point. A study of *Echinarachnius* metaphase figures, fixed in picro-acetic, shows that most of the asters contain no granules; a few have one, or two, or three, or more. The granules vary as to number, as to size, and as to location in the "centrosome"; the situation concerning granules in one aster of a metaphase figure, gives no clue whatever concerning the condition existing in the other aster. Hence in *Echinarachnius* eggs, configurations occur in a very limited number that exactly duplicate Boveri's illustrations of *Echinus* central body structure, having one or two "centrioles" surrounded by a "centrosome.' Such configurations, however, are without any significance. It will require a study of *Echinus* eggs by the methods of the present investigation to prove whether or not the condition existing in *Echinarachnius* applies to *Echinus*. It is highly probable, however, that the factors mentioned above, *i.e.*, coagulation artifacts of the astral center, and the random occurrence of cytoplasmic granules, explain not only Boveri's observations but also the other types of echinoderm central bodies formerly illustrated.

That type of central body, previously described, that is composed of a minute centriole only (Fig. 32), is probably explained by a misinterpretation of a cytoplasmic granule occurring at the center of an aster, that is fixed by a reagent which does not produce a demarked centrosome.

The type of central body composed of a large "centrosome" only, without a centriole, occurs in two major classes, each of which shows many modifications: the first type is definitely delimited, having a mulberry-like or pleuricorpuscular structure, and is darkly stained (Fig. 33); the second type is larger and less clearly delimited, having a vacular or reticular structure, and is lightly stained (Fig. 34). The center of the latter type is in some cases practically empty as though the substance had been dissolved away. These two classes are well illustrated in Wilson's study of *Toxopneustes*. In his earlier work ('95) he uses eighty parts of a saturated solution of mercuric chloride, with twenty parts of glacial acetic acid. In his later work ('00) a sublimate-acetic mixture is again used, but in this case there is a smaller percentage

of acetic acid.[6] It is observed that the larger quantity of acetic acid produces the large, lightly-stained vacular "centrosome" (Fig. 34); whereas the lesser amount produces the dark, small pleuricorpuscular type (Fig. 33). This situation is exactly duplicated in the case of *Echinarachnius*, where a sublimate-acetic mixture containing twenty per cent. acetic acid produces a light vacular center (Fig. 6), whereas one containing but one per cent. acetic acid produces a dark pleuricorpuscular center (Fig. 3). This case is but another illustration of the ability of the fixing agent to modify the coagulated central body structure.

Thus it appears that previous studies of echinoderm central bodies are probably explained by the present investigation, although the possibility of the effect of species differences must be kept in mind. When the various types of central bodies formerly described (Figs. 28–34) are analyzed in the light of the present study, it seems highly probable that the phenomena are due to two fallacies: first, the so-called centrosomes are actually coagulation products of the focal point of well formed rays; second, the so-called centrioles are actually cytoplasmic granules that occur sporadically at the astral center.[7] This situation completely invalidates the usual hypotheses concerning central bodies in echinoderms, a review of which has been given previously (Fry, '29, pp. 101–104). *If future study of echinoderm central bodies in various species shows that the phenomena are like those in Echinarachnius, it will prove, at least in this phylum, that the current central body hypotheses are actually based upon misinterpretations of coagulation artifacts and cytoplasmic granules.* The reasons have been given previously (Fry, '29, pp. 121–123) why it is probable that this conclusion may be found to have wide application to central bodies in fertilization and mitosis generally.

The wide differences between the conclusion of former investigations and that of the present study concerning the significance of echinoderm "central bodies," is explained by the equally wide difference between the method of study used by former inves-

[6] This sublimate-acetic mixture contained one or two per cent. glacial acetic acid according to information received from Prof. Wilson.

[7] A third source of error in early sperm-asters is a misinterpretation (Boveri, '00) of the bi-lobed granule of the sperm's middle-piece (cf. Fry, '29, p. 105).

tigators and that of the present work (Fry, '28, pp. 387–392; '29, pp. 109–113). The usual cytological procedure is to use a limited number of fixatives and to select certain of the coagulation products as "normal," dismissing the others as "poorly fixed," *without a sufficiently complete and quantitative consideration of all possible classes of coagulation products.* Both methods have the same aim, that of determining which coagulation products are "normal," *i.e.*, which of them most nearly approximate the living condition. The methods differ concerning the completeness of the analysis necessary to decide what is "normal." The old method is open to the danger that the "normal" is determined upon the basis of incomplete and partial data. The present method makes a more complete analysis. It is not a superficial study of many classes of coagulation products, in contrast to an intensive study of a few "best" types; it is an equally intensive study of all types, omitting none, in order to determine what is "normal."

The prophecy is made that if future study of cellular components is carried on by the simple quantitative method used in the present work, that various cytological facts, now assumed to be based on sound evidence, will be found actually to be based upon certain selected groups of coagulation products, without a sufficient consideration of their quantitative relationships in relation to other classes. Results may possibly be attained in various fields, as significant as are those here reported concerning the so-called central bodies in echinoderm fertilization.

Résumé.

1. When metaphase asters of the first cleavage division in *Echinarachnius* eggs are fixed in a wide variety of reagents, central bodies (centrosomes) are present only in those cases where the fixative clearly coagulates rays. They are absent when astral rays are fixed vaguely or not at all.

2. This result is in harmony with that of the preceding studies (Fry, '28, '29). Hence whether astral structure is modified by the various stages in the normal mitotic cycle, or by various fixatives: whether this occurs in nuclear asters of fertilized eggs or in cytasters of artificially activated eggs; central bodies (centrosomes) are present only when clearly fixed rays reach the astral

center. This indicates that they are nothing but coagulation artifacts of the focal point of well formed rays and that they have no existence as individualized bodies in the living condition.

3. It is probable that the phenomena in *Echinarachnius* are typical of echinoderms generally. The illustrations in previous studies of echinoderm central bodies fall into three classes: (1) those composed of a centriole which is a minute period-like granule, surrounded by a centrosome which is a larger, more diffuse structure; (2) those composed of the minute centriole only; (3) those composed of the larger centrosome only. The present investigation indicates that the supposed "centrosomes" are actually coagulation artifacts of the focal point of well formed rays, and that the supposed "centrioles" are actually cytoplasmic granules that have a random occurrence at the mid-point of asters. These facts completely invalidate the usual hypotheses concerning central bodies in echinoderms.

4. It is probable that this conclusion may have wide application to central bodies in fertilization and mitosis generally. Whether or not such is the case requires further study of various species in the various phyla, by the same quantitative method used in the present investigation.

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THE SO-CALLED CENTRAL BODIES IN FERTILIZED *ECHINARACHNIUS* EGGS.

III. The Relationship between Central Bodies and Astral Structure as Modified by Temperature.[1]

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Introduction.

Two previous studies have demonstrated that the supposed central bodies in fertilized *Echinarachnius* eggs are actually nothing but coagulation artifacts of the focal point of clearly fixed rays, misinterpreted as "centrosomes," plus the occurrence of cytoplasmic granules at the astral center, misinterpreted as "centrioles." This conclusion renders meaningless the usual hypotheses concerning echinoderm central bodies, *i.e.*, that they are individualized structures which are the formative foci of asters, and that they may have genetic continuity from cell to cell and from generation to generation.[2]

In the first of these studies (Fry, '29*a*) the fixative and the environment are constant. Modifications in astral structure occur during different stages in the normal mitotic cycle: centrosomes

[1] The material was secured at the Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] A review of the literature concerning central bodies in echinoderm fertilization, together with a brief discussion of terminology, will be found in the earlier papers of this group (Fry, '29*a*, pp. 101–109; Fry, '29*b*, p. 132 and pp. 143–147).

are present only during the middle history of sperm- and cleavage-asters when rays are clear; they are absent at other times when rays are vague. In the second study (Fry, '29*b*) the astral stage (metaphase) and the environment are constant. Metaphase astral structure is modified by the different coagulation effects of various fixatives: centrosomes are present only in the case of those reagents that clearly coagulate rays; they are absent when rays are fixed vaguely or not at all. In the present study the astral stage and the fixative are constant. Astral structure is modified by changes in the environment with reference to temperature.

If the conclusion established by the previous studies is correct, that *Echinarachnius* central bodies (centrosomes) are actually coagulation artifacts of the inner ends of clearly fixed rays, then in the present study they should occur only at those temperatures at which rays are well formed in the living condition, and are clearly fixed when a proper reagent is used: they should be absent at those temperatures when rays are vaguely formed in the living egg, and hence are vaguely fixed.

The various sets of eggs were fertilized at 18° C. Within a minute or so after insemination each set was transferred to sea water of a given temperature. Sets were run at two degree intervals from 4° C. to 28° C. There is a very low percentage of development at 4° C., hence there are no experiments at lower temperatures. Eggs cytolyze at 28° C., hence this is the upper temperature limit. The temperature of each set did not fluctuate more than two tenths of a degree.

Eggs were fixed in a sublimate-acetic mixture (saturated solution of mercuric chloride, 97.5 per cent.; glacial acetic acid, 2.5 per cent.). This fixative is commonly used in the cytological study of echinoderm eggs. It is a reagent that coagulates rays clearly. Eggs were sectioned at 5 μ thick and stained with Heidenhain's haematoxylin. They were studied at 750 $\times$ magnification.

At each two degree temperature interval, about twenty-five metaphase and about twenty-five anaphase figures were each measured and analyzed with reference to the twenty-two points previously listed for the study of cleavage asters (Fry, '29*b*, Table I., p. 135). Only such eggs were selected which happen to be sectioned in a plane more or less parallel to that of the elongation

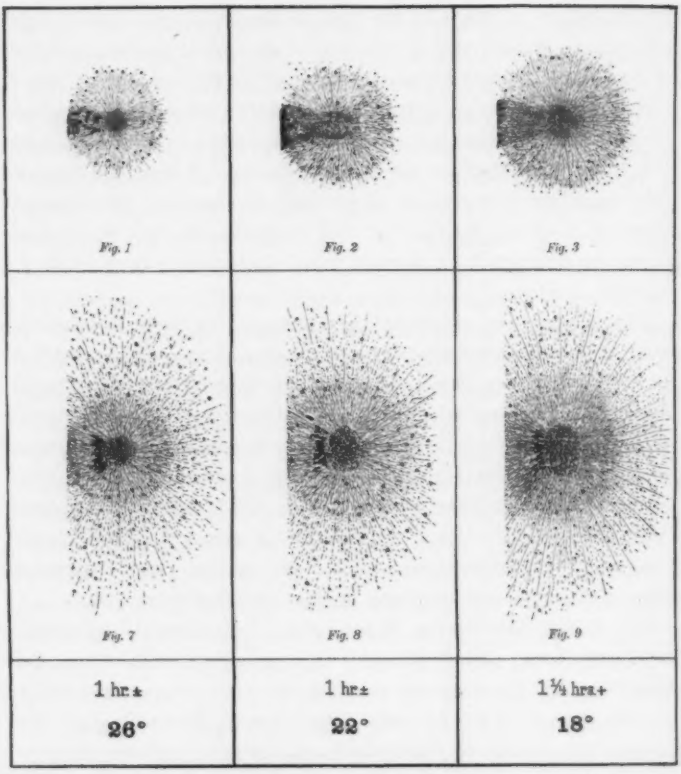
of the mitotic figure. In the case of the metaphase asters, only those were studied that have rays of maximum clarity, and the chromosomes aligned in two groups ready to separate (*i.e.*, "late" metaphase asters), in contrast to a slightly earlier stage in which the rays are less clear and the chromosomes are in a tangled mass (*i.e.*, "early" metaphase asters). In the case of anaphase figures, only those were studied in which the chromosomes have passed to the end of the spindle but still maintain the typical elongate form, in contrast to a slightly later stage when they exist as rounded vesicles.

The figures (pp. 154–155) are arranged in vertical columns, each of which illustrates the metaphase and anaphase condition at a given temperature. Although the material was fixed and studied at two degree intervals, the illustrations show the phenomena only at four degree intervals, since such procedure adequately presents the facts. In the vertical column for each temperature, the metaphase aster is shown above, with the anaphase aster below it. For each temperature is given the approximate time after fertilization required for the eggs to reach that stage when metaphase and anaphase figures are most numerous.

The illustrations are 650 × enlarged. For example, the coagulated metaphase aster at 18° C. (Fig. 3) is 32 μ in diameter; the dimensions of the anaphase aster at the same temperature (Fig. 9) are 70 μ × 35 μ. In each figure, every dimension and the physical appearance of each part is an average of all the observations made in about twenty-five asters belonging to that class. The chromosomes, however, are an exception to this; they were studied only superficially. The method of study used in the present work is the same as that previously described (Fry, '28, pp. 387–392; '29*a*, pp. 109–113).

The Effects of Various Temperatures upon the Relationship between Clearly Fixed Rays and Central Bodies in Cleavage Asters of Fertilized *Echinarachnius* Eggs.

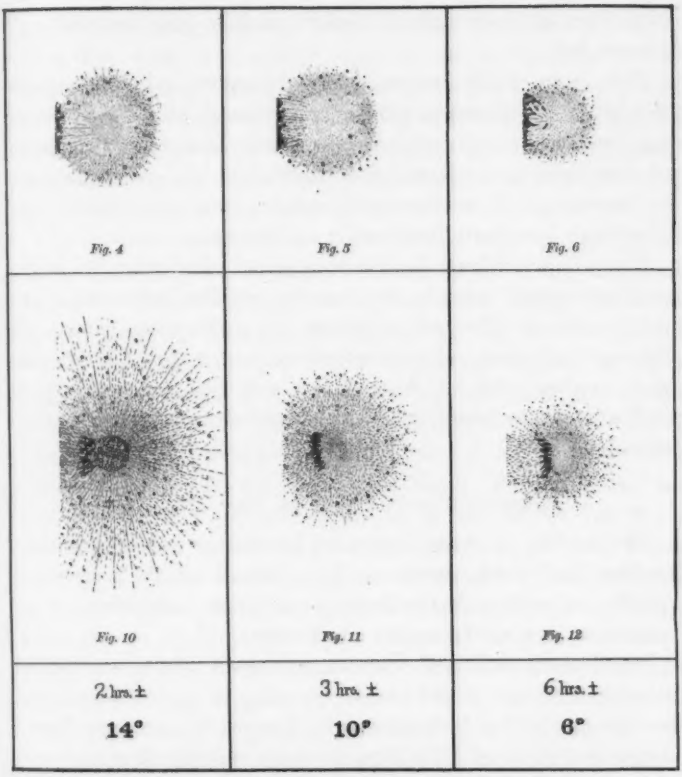
A study of the figures shows that in metaphase asters a well formed pleuricorpuscular central body (centrosome) is present only at temperatures from 26° C. to 18° C., when rays are clear



THE EFFECTS OF VARIOUS TEMPERATURES UPON THE CENTROSOMES IN CLEAVAGE ASTERS

The figures are arranged in vertical columns with reference to four degree temperature intervals. Metaphase asters are above; anaphase asters are below. In each figure each dimension and the physical structure of each part, is an average of the observations made in about twenty-five division figures, according to the points listed, Fry, '29*b*, Table I., p. 135. The chromosomes, however, were studied only superficially. The figures are

(Figs. 1–3). The rays begin to fade at 14° C., and the central body is much less definite (Fig. 4). From 10° C. to 6° C. the rays are very vague and there is no central body (Figs. 5–6). The same situation holds in anaphase asters with the exception that the rays fade at a somewhat lower temperature than is the



RELATIONSHIP BETWEEN CLEARLY FIXED RAYS AND OF FERTILIZED *ECHINARACHNIUS* EGGS.

650 × enlarged. The fixative is a saturated solution of mercuric chloride, 97.5 per cent., plus glacial acetic acid, 2.5 per cent. Central bodies (centrosomes) are present at the upper temperatures where rays are clear. They fade out as the rays become vague at the lower temperatures. This indicates that the so-called centrosomes are coagulation artifacts of the focal point of clearly fixed rays. There are no centrioles.

case in the metaphase stage. In both cases the central bodies disappear simultaneously with the fading of clear rays. This result is in harmony with that obtained in a study of the effect of various environmental factors upon cytasters in artificially activated eggs (Fry, '28, Chart II.), where central bodies occur

only when the environmental factors produce cytasters with well formed rays.

This study yields further evidence supporting the conclusion that the so-called central bodies (centrosomes) of *Echinarachnius* eggs are coagulation artifacts. No matter how the ray structure of coagulated asters is modified, whether by the astral stage, or by fixation, or by environmental agents, centrosomes occur only when rays are clearly fixed and reach the center.

There is no evidence for the presence of centrioles. As in the previous studies, cytoplasmic granules are abundant throughout the cytoplasm. They are numerous among the outer portion of the rays, but occur only sporadically in the centrosome, differing as to number, size, and location. A very limited percentage of such granule configurations simulate centrioles, but they are without significance.

Observations Concerning the Spindle.

The method of study here used concerning the central body problem also yields significant facts concerning the asters and spindles, as well as the interrelations of these components of the achromatic part of the mitotic mechanism.

This investigation yields data concerning the rôle of the spindle in contrast to that of the asters. Keeping in mind the fact that but one fixative has been used, it is observed in metaphase asters that at 26° C. to 18° C. (Figs. 1–3) the spindle fibers are very numerous and lie close together, whereas at 14° C. to 6° C. (Figs. 4–6) they are less numerous and lie farther apart. Nevertheless, at the lower temperatures, the spindle maintains its identity in a manner not shared by the asters. At 6° C. (Fig. 6) the metaphase aster is very small, and is composed of exceedingly vague rays in contrast to the large clear-rayed aster occurring at 18° C. (Fig. 3); whereas at 6° C. the spindle maintains the same width and roughly occupies the same area as it does at the higher temperatures. It seems, therefore, that the spindle is that part of the achromatic mechanism least effected by temperature changes. The significance of this fact requires further study.

It is to be remembered that the present paper is an analysis of the effects of temperature upon only a small part of the astral

cycle, when using but one fixative. It is necessary to supplement this work by a similar study of the mitotic figure at all stages from fertilization to first cleavage; to duplicate the method of study in a variety of fixatives; and to consider the cytological observations with reference to percentages of normal and abnormal development occurring at each temperature. Such a procedure may yield valuable data concerning the division figure.

The previous study of the effect of fixatives (Fry, '29*b*) yields facts concerning the structure of the spindle. With the exception of several coagulation products in which neither the asters nor the spindle are fixed (Figs. 7, 9, 13 and 21), the spindle shows a structure more definitely fibrous than that of the astral rays. In cases where the rays are coarsely fixed, the spindle fibers are even more definite; in cases where the rays are delicately fixed the spindle fibers stand out far more distinctly; in cases where the rays are practically unfixed (Figs. 1, 17 and 24) the spindle fibers are quite clear. It is well known that in the living condition the spindle generally appears to be structureless, whereas the aster shows a radial arrangement. Since in practically all fixatives the spindle appears to be more definitely fibrous than the ray configuration of the aster, this indicates that such is actually the case in the living condition. Such a definite linear organization can scarcely be regarded as a coagulation artifact since it occurs in so many diverse fixatives. This appears to be an instance where the fixed material yields data more dependable than does a study of the living condition.

Résumé.

1. When fertilized *Echinarachnius* eggs are allowed to develop at various temperatures, and are fixed in a sublimate-acetic mixture, central bodies (centrosomes) are present in metaphase and anaphase asters only at the upper temperature intervals where rays are well formed. Simultaneously with the fading of the rays at low temperatures, the centrosomes disappear.

2. This result is in harmony with that of the previous studies of this group. Whether the structure of fertilization asters, or of cytasters, is modified by the various stages in the normal mitotic cycle, or by fixation, or by modifications of the environment,

centrosomes are present only when clearly fixed rays reach the astral center. They are therefore nothing but coagulation artifacts of the focal point of well formed rays, having no existence as individualized bodies in the living condition.

3. There is no evidence for the presence of centrioles. They are occasionally simulated, however, by cytoplasmic granules.

4. The method of study used in this group of papers also yields significant data concerning the mitotic mechanism as a whole. The spindle has a structure more clearly fibrous than is the ray configuration of the asters. Under the influence of low temperatures the spindle maintains its typical dimensions in contrast to the behavior of the asters which almost disappear.

LITERATURE CITED.

A bibliography of the literature concerning central bodies in echinoderm fertilization will be found in the first and second papers of this group (Fry, '29*a*; '29*b*). A list of those papers belonging to the present series of studies follows:

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'28 Conditions Determining the Origin and Behavior of Central Bodies in Cytasters of *Echinarachnius* Eggs. BIOL. BULL., 54.

'29*a* The So-called Central Bodies in Fertilized *Echinarachnius* Eggs. I. The Relationship between Central Bodies and Astral Structure as Modified by Various Mitotic Phases. BIOL. BULL., 56.

'29*b* The So-called Central Bodies in Fertilized *Echinarachnius* Eggs. II. The Relationship between Central Bodies and Astral Structure as Modified by Various Fixatives. BIOL. BULL., 57.

CLEAVAGE RATES IN FRAGMENTS OF CENTRIFUGED *ARBACIA* EGGS.

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In order to test whether the granules in the sea urchin egg affect the rate of cleavage, *Arbacia* eggs were centrifuged, and before appreciable redistribution of the stratified materials could take place, were cut along the plane of stratification into two approximately equal halves. One half was practically free from granules, the other contained practically all of them. The two halves of each egg were subjected to the same degree of injury in cutting, since they were the result of one operation, and they were then placed together in a small glass dish in a moist chamber, and were inseminated.

The eggs of *Arbacia* contain an abundance of granular material, especially pigment, and they can readily be stratified with the centrifuge. The two halves of the centrifuged egg differ markedly in viscosity as well as in granular content. The clear half is considerably less viscious than the normal egg, and the granular half is considerably more so.

On account of the difficulties of operation, and of the high mortality, especially of fragments of centrifuged eggs, together with limited time in which to work, not enough cases of all sorts were obtained to make the actual average times recorded in the results highly significant, but certain relationships are significantly revealed.

METHOD.

Stratifying and Cutting.

Arbacia eggs are completely stratified by centrifuging for twelve minutes at about 1,350 gravities (2,600 r.p.m. with 18½ cm. radius). The materials of the egg which are moved by the centrifuge come to lie in four sharply defined specific gravity zones. The smallest and lightest of these is a small oil cap composed of comparatively large droplets. The next zone is clear and sup-

posedly watery. It is seen by oil immersion lens and dark field illumination to contain very few particles which are not colloidal, or nearly so. The egg nucleus lies in this watery zone just under the oil cap. The third and largest zone contains the bulk of the granular material of the egg including the yolk and the finer particles of pigment. The fourth and densest zone contains most of the pigment collected in a cap which is less sharply set off from the third zone than are the other zones from each other.

When the egg is completely stratified the plane of contact of the watery zone and the large granular zone passes near the equator of the egg but slightly toward the oil cap pole, so that the granular part comprises a little more than half of the egg. By means of a Taylor micro-manipulator eggs were cut into two fragments of approximately equal size, by two types of cuts: (1) Some were cut along the equator of the egg in which case the fragments were of equal size but the "clear" fragment contained a slight amount of granular material, and (2) others were cut along the plane of contact of the zones in which case the "clear" fragment was slightly smaller than the "granular." Fragments from these two types of cuts are grouped indifferently together in Table I. because no difference was found in the result.

In all cases the eggs were cut within twenty minutes after centrifuging. In uncut eggs redistribution of the stratified materials can be seen to have started half an hour after centrifuging.

After cutting, the fragments were not inseminated until ten minutes had passed. If then moderately weak sperm suspensions were used, polyspermy was almost entirely avoided. If the fragments were inseminated immediately after cutting polyspermy occurred oftener than not. The ten minute interval after cutting apparently allows the membrane to recover and repair and like the normal egg surface it becomes impervious to sperm after fertilization.

Nuclear Content.

Since the egg nucleus always lies in the watery zone of the centrifuged egg, it is always contained in the fragment which we refer to as "clear." Consequently, after fertilization the "clear" fragment is diploid and the "granular" is haploid.[1] It is known

[1] At least in the early cleavage stages.

that haploid and diploid fragments of normal non-centrifuged Echinoderm eggs cleave at different rates. (Tennent, Taylor, Whitaker, 1929.) The diploid fragments cleave first. As a control, the time interval from insemination until the first cleavage of nucleated and non-nucleated fragments of normal non-centrifuged eggs was first measured. Eggs from the same female, and at the same temperature, were stratified and cut, so that on the difference of "haploid" and "diploid" the difference of "clear" and "granular" was superimposed. The added effect of this second condition was taken as the measure of the difference in cleavage rate due to approximately double the normal concentration of granules in one fragment and to an almost complete absence of granules from the other. A significant measure is furnished by a comparison of the difference in cleavage rate of haploid and diploid fragments of non-centrifuged eggs with the difference between haploid ("granular") and diploid ("clear") fragments of centrifuged eggs.

Temperature.

The temperature for a particular experiment was approximately constant. Moist chambers were filled from the main salt water supply of the laboratory and were set aside together. After equilibrium had been reached with room temperature (as indicated by a thermometer) the temperature was recorded. It was usually slightly lower than room temperature due to a certain amount of evaporation from the outside of the glassware. During the course of the experiment, which lasted from forty minutes to an hour and a quarter, the temperature did not vary more than $\pm\frac{1}{4}^\circ$ C. But the two halves of the same egg, being compared for cleavage rate, were kept much closer yet to the same temperature as they were in the same dish of water, lying within a millimeter of each other, from the time of insemination until both fragments cleaved. The room temperature varied at different times and on different days so that the water temperatures ranged between 20° and $24\frac{1}{2}^\circ$ as indicated in Table I.

Condition of Material.

The experiments were performed during the last two weeks of July, 1928. Eggs and sperm were in excellent condition. In all, the eggs of twenty different females were used; but many more females were discarded after preliminary tests. For it was found that even among eggs of which more than 95 per cent. develop good fertilization membranes, there is great variation in aptitude to cutting. This is especially marked after centrifuging. The eggs of some females tend to burst with the slightest provocation, while those of others can be cut with the greatest ease. For the present purpose it was of great importance to minimize injury to the fragments. This was largely realized by taking care in selecting suitable eggs, and in avoiding pressure and squeezing, and by discarding fragments known to be damaged.

Results.

Explanation of Table I.

In the following table each different female has a number which for convenience has been assigned in the order of temperature sequence. The temperature indicated for each female applies within the limits stated, to all eggs taken from her: controls, centrifuged controls, and fragments of both non-centrifuged and centrifuged eggs. Time is counted to the nearest half minute. In the control dishes of both the centrifuged and non-centrifuged eggs time is counted from insemination until fifty per cent. of the eggs in the microscope field (usually about twenty-five) have just cleaved. But in the case of the fragments, the recorded time is the observed time for each particular fragment. A certain stage which could be repeatedly recognized was arbitrarily chosen and counted as the time of cleavage. This stage occurs just as the furrow has passed completely through the egg, and it is reached about one minute after the start of cleavage. In selecting this basis for counting time it was not forseen that the rate of the passage of the furrow would be appreciably altered in many of the fragments of the centrifuged eggs. When this phenomenon became apparent, the time of cleavage for such fragments, as recorded in Table I., was then taken as one minute after the first appearance of the cleavage furrow.

Each set of figures in the three columns, "haploid," "diploid," "difference" refers to the two halves of the same egg. In cases where one of the two fragments failed to yield a result a dash is put in its place and also in the "difference" column.

TABLE I.

TIME TO CLEAVAGE IN MINUTES.

Female.	Temperature ± ¼° C.	Non-Centrifuged Controls 50% Cleaved.	Centrifuged Controls 50% Cleaved.	Non-Centrifuged Fragments.			Centrifuged Fragments.		
				Diploid.	Haploid.	Difference.	Diploid.	Haploid.	Difference.
1	20°	56.0		57.0	65.5	8.5			
		57.0		57.0	65.5	8.5			
				59.0	66.0	7.0			
				60.0	67.0	7.0			
2	21°	56.5		55.5	61.5	6.0			
				56.5	61.5	5.0			
				58.5	64.5	6.0			
				58.5	64.5	6.0			
				59.0	65.5	6.5			
3	22°	52.0					56.0	—	—
							52.0	—	—
							45.0	—	—
4	22°	52.0	52.0	46.5	52.0	5.5	49.0	56.0	7.0
				46.0	53.0	7.0	48.0	—	—
5	22°			51.0	60.0	9.0			
6	22¼°	50.0	49.5				45.0	53.0	8.0
							49.0	56.0	7.0
7	22¼°	49.0					53.0	58.0	5.0
							48.0	56.0	8.0
8	22½°	50.0		45.0	51.0	6.0			
				48.0	53.0	5.0			
				46.0	51.5	5.5			
				53.0	59.0	6.0			
				48.0	—	—			
				49.0	61.0	13.0			
				48.0	55.5	6.5			
				47.0	53.0	6.0			
				49.0	53.5	4.5			
				48.0	—	—			
				49.0	52.5	3.5			
				48.0	53.0	5.0			
				47.0	—	—			
				47.5	—	—			
				48.5	52.5	4.0			
9	22½°	50.5							
10	22½°	49.0	50.0	46.5	55.5	9.0	46.0	51.5	5.5
				48.5	54.5	6.0	—	54.0	—
							48.0	52.5	4.5

Female.	Temperature ± ½° C.	Non-Centrifuged Controls 50% Cleaved.	Centrifuged Controls 50% Cleaved.	Non-Centrifuged Fragments.			Centrifuged Fragments.		
				Diploid.	Haploid.	Difference.	Diploid.	Haploid.	Difference.
							48.0	—	—
11	22½°	49.5		48.0	54.0	6.0	51.5	—	—
				45.0	52.0	7.0	51.5	59.0	7.5
				45.5	53.5	8.0			
				50.0	56.0	6.0			
				48.0	55.0	7.0			
				55.0	60.5	5.5			
12	22¾°	48.0	47.0				45.0	51.0	6.0
							48.5	—	—
							45.0	52.0	7.0
							44.5	53.0	8.5
							—	51.0	—
							47.5	—	—
							46.0	54.0	8.0
13	23°	49.0							
		49.0							
14	23°	43.5	43.5				—	51.0	—
							46.0	55.0	9.0
15	23°	46.0	47.0				43.0	51.0	8.0
16	23½°	44.5	45.5	42.0	48.5	6.5	46.0	52.0	6.0
		44.5		42.0	—	—	42.0	49.0	7.0
				43.0	50.0	7.0	44.0	50.0	6.0
							47.0	50.0	3.0
17	23½°	46.0	48.0	46.0	52.0	6.0	42.0	49.0	7.0
				45.0	50.0	5.0	52.0	57.0	5.0
							44.0	51.0	7.0
							43.0	50.0	7.0
18	23½°	46.0	46.0				49.5	—	—
							55.5	63.5	8.0
							45.5	52.0	6.5
							48.5	56.5	8.0
19	24°	44.0	46.0	43.0	53.0	10.0			
				49.5	55.5	6.0			
				44.0	48.5	4.5			
20	24½°	43.0							
		41.0							
					Average	6.5		Average	6.8

Effect of the Stratified Materials.

It is observed in Table I. that the averages of the difference in time from fertilization to cleavage of the diploid and the haploid fragments in the two cases, non-centrifuged and centrifuged, are practically the same: 6.5 minutes (38 cases) for the non-centrifuged, and 6.8 minutes (25 cases) for the centrifuged. While there are not enough cases to establish a negligible probable error, it is evident that the effect of the granules on the cleavage rate is either very slight or entirely absent.

In averaging these differences in cleavage rate the differences in temperature of the different experiments have been ignored, because in all cases the two fragments compared were from the same egg and at the same temperature. Therefore while the actual time lapse to cleavage varies considerably within the 4½° temperature range, as seen in Table I., both fragments are speeded up or slowed down so that the difference is probably not changed within the limits with which we are at present concerned, considering that the averages compared are derived from fairly comparable distributions over the temperature range.

The difference between the cleavage rate of haploid and diploid fragments is practically the same in centrifuged and non-centrifuged eggs. But it is still possible that the two fragments of the centrifuged egg, for example, are both delayed almost exactly the same amount compared with those of the non-centrifuged. In this case the differences between haploid and diploid fragments in the two cases would still be the same. The diploid centrifuged fragment might conceivably be delayed by a shortage of granules and the haploid centrifuged fragment by increased viscosity. If this were the case, the results would indicate such an exact compensation from independent causes that such an equal delay does not seem likely. The direct comparison must be made with cases in which the temperature is the same throughout within close limits. This condition is realized in eggs from a particular female. The cases which best fulfil the requirements are those from females 4, 10, 11, 16, and 17 (Table I.). In each of these five cases the averages are taken of each the haploid and the diploid centrifuged and the haploid and the diploid non-centrifuged fragments. The five averages of the haploid centrifuged fragments from the five females are then averaged, thus giving equal weight to each temperature. In the same way the other three sets (diploid centrifuged, haploid non-centrifuged, and diploid non-centrifuged), are also averaged. In this way error from unequal distribution over the temperature range is avoided. The results are: average time to cleavage of haploid fragments from non-centrifuged eggs, 52.6 minutes (based on 14 cases); haploid fragments from centrifuged eggs, 53.9 minutes (13 cases); diploid fragments from non-centrifuged eggs, 46.0 minutes (15 cases); diploid fragments from

centrifuged eggs, 47.4 minutes (15 cases). This shows a slight delay on the part of both fragments of the centrifuged egg but not much greater than is found for whole centrifuged eggs (see below) and not as great as the limits of error for so few cases. Here we are comparing fragments from different eggs. A sample of eggs has a probability distribution of time lapse to cleavage covering several minutes. Hence from this we can only conclude that any delay of both fragments of the centrifuged egg is at least very slight.

From the ten females numbered 4, 6, 10, 12, 14, 15, 16, 17, 18, 19, centrifuged and non-centrifuged controls, both at the same temperature, were timed for 50 per cent. cleavage. The average time is 46.9 minutes for non-centrifuged, 47.4 minutes for centrifuged eggs.

Effect of Amount of Nuclear Material

It has been shown that diploid fragments cleave sooner than haploid fragments. This seems to indicate that the ratio between nuclear and cytoplasmic amounts is a determining factor in cleavage rate. But since the sperm contributes a precursor to the amphiaster as well as a nucleus it may be that the derivatives of the sperm are not exactly equivalent to the egg nucleus in effect on cleavage rate. A comparison between the cleavage rates of normal uncut diploid controls and diploid fragments bears on this point. Here the nuclear content is identical, consisting of an egg and a sperm nucleus, but the amount of cytoplasm is half in the case of the fragment. For this comparison it is necessary to have approximately constant temperature, so two large groups of cases will be selected within which the temperature range is small. The first group includes results from females 3 to 11 inclusive. The temperature range is 22° C. to 22½° C. The second group includes females 12 to 18 inclusive, with temperature range from 22¾° C. to 23½° C. The results are shown in Table II. It is seen that diploid fragments cleave sooner than normal eggs in spite of the injury of cutting.

The viscosity of the two fragments "clear" and "granular" is found to differ markedly, but no quantitative measure of this difference has been made. The evidence for difference in viscosity.

and the results and implications are considered in the latter part of the last section (Discussion).

TABLE II.

Cases.	Group I 22°–22½°	Average time (minutes)
11	whole egg controls (50% cleaved)	50.3
40	diploid fragments	48.3
31	haploid fragments	54.8
	Group II 22¾°–23⅓°	
15	whole egg controls (50% cleaved)	46.2
25	diploid fragments	45.7
23	haploid fragments	52.1

Development.

Lyon (1906) found that stratified *Arbacia* eggs develop normally, and Morgan and Spooner (1909) found that they do so in accordance with the original polarity of the egg and without regard to the planes of stratification of the egg or the concentrated zones of materials which are moved by the centrifuge.

In the case of uncut eggs it can be readily observed that a large amount of concentrated material is redistributed in most of the eggs within three quarters of an hour. If they are fertilized the redistribution before cleavage is even more pronounced, aided no doubt by the protoplasmic flows which precede cleavage. It is entirely possible that fragments from eggs cut immediately after centrifuging would show developmental alterations not present in uncut eggs. But time was not available during the present work to follow the development of these fragments. Several early blastulae were incidentally observed from each kind of fragment but they were not followed further. However fragments from centrifuged eggs of the Pacific coast starfish *Patiria miniata* have been followed. These eggs cannot be stratified as sharply as Arbacia eggs, but after 35 minutes at 4,000 r.p.m. with 20 cm. radius (3,600 gravities) the eggs of most females become quite clear at one end. Complete gastrulae have been reared from clear fragments as well as from their mates. In this case there is a complication, which will not be dealt with here, concerning localized

formative stuff (Whitaker, 1928). There is also an unusual nuclear phenomenon in this egg. The nuclei from the polar bodies move back into the egg when it is centrifuged and form a polyploid egg which divides to 6 or 8 cells a little before the normal egg divides to 2 cells. The behavior of these nuclei will be considered at another time.

Discussion.

Cleavage and Oxidation.

Warburg (1913) found that when unfertilized sea urchin eggs are ground in a mortar with sand, oxygen consumption continues independently of the living condition for some hours at about the same rate as in normal unfertilized eggs. He found further (1914) that if eggs with membranes broken by shaking are centrifuged so that the granules are concentrated, about 82 per cent. of the oxidation takes place in the sediment containing the granules.

If the granules in the living egg act as catalysts of oxidation as in the crushed eggs, it seemed possible that a difference in developmental rate might be found in the two fragments, granular and non-granular. On the other hand it has been known for some time that developmental rate[2] and oxidation rate do not proceed very closely together. Crozier (1925) points out that the two processes are essentially dissimilar since development must depend on synthetic processes. It is not then to be expected that oxidation rate is normally the limiting factor in cleavage rate. But it may become so experimentally, as shown for example by Loeb (1896) when he suppressed cleavage in sea urchin eggs by complete absence of oxygen. Therefore, if the granules are catalysts of oxidations, if their concentration were sufficiently diminished the oxidations which they catalyze might become limiting to cleavage rate. Such an effect was looked for especially in the "clear" fragments of completely stratified eggs. But such limitation was not found. Even when excluded to a very great extent the granular material of the egg does not become limiting to cleavage rate;

[2] Distinction should be made between cleavage rate and growth rate in the sense of increase in weight. In the early cleavage stages weight is in all probability lost, except for the intake of water since CO_2 and heat are produced and no food is taken in. However synthetic processes are taking place since embryological differentiation is going on.

nor does it appreciably alter cleavage rate when its concentration is approximately doubled.

Amberson (1928) has recently published results on the effect of reduced oxygen pressure on cleavage rate in *Arbacia* eggs. He found that the oxygen pressure must be reduced to 20 mm. Hg. before the rate of oxygen consumption in fertilized *Arbacia* eggs begins to decline sharply. But not until the oxygen pressure is further reduced to 11 mm. Hg. is the cleavage rate retarded. At this pressure the oxygen consumption is reduced to about one half normal. Cleavage is suppressed completely at about 4 mm. Hg. These results prove directly that oxygen consumption must be reduced to about one half before it becomes limiting to the reactions which lead to cleavage.

If as much as 80 per cent. of the oxidation takes place in the granular half of the egg, it must be reduced to 20 per cent. in the "clear" fragment. This 20 per cent. is less than half normal (*i.e.* half of 50 per cent.). From Amberson's results, which were not published until after my own work was done, it can be estimated that the "clear" fragment ought to show retardation of cleavage rate, if oxygen consumption is reduced to less than half by the removal of the granules.

Viscosity.

The two halves of the cut centrifuged egg differ in viscosity as well as in content of formed bodies. The pigmented half is considerably more viscous as shown in several ways. After the egg is cut, the "clear" fragments round up more quickly than the "granular." When fragments are ruptured, long extensions flow out from "clear" fragments while smooth bulges result on "granular" fragments. A good rough comparative measure, in spite of the danger of subjective interpretation, can be made by prodding with the needle and observing the rate of flow in response to distortion. It can be concluded with reliability that there is a gradient of viscosity perpendicular to the strata with a maximum in the pigment cap.

It has been shown that the amount of granular material contained in the fragment has practically no effect on the time lapse until cleavage starts. But there is a marked difference between

"clear" and "granular" fragments in the time required for the act of cleavage to be completed. The "clear" liquid fragment cleaves completely in less than a minute, often in less than half a minute. It does so more quickly than the normal egg. The pigmented viscous fragment takes from one or two to six minutes or sometimes more to cleave. The furrow usually starts on the least viscous side of the pigmented fragment and follows slowly along the gradient of viscosity, *i.e.*, passes through the pigmented cap last. This suggests a connection between the viscosity and the rate of passage of the cleavage furrow (perhaps by an effect on the mechanics of cleavage). But it is significant that the rate of the steps leading to the start of cleavage is not appreciably affected. The precleavage processes are neither retarded nor accelerated by change of viscosity within the limits of change brought about in these experiments. The exact nature of the gradient of viscosity is not known. There is no evidence to show how much it is due to a mere mechanical displacement of protoplasm by more solid particles or to what extent it is due to transference of materials taking part in a more truly viscous gel structure.

Summary and Conclusion.

1. Fragments of centrifuged *Arbacia* eggs, "clear" and "granular," start to cleave at practically the same time after fertilization as fragments of non-centrifuged eggs.

2. The cleavage furrow passes more slowly through the "granular" fragments than through the "clear" fragments. It passes through the fragments of non-centrifuged eggs at an intermediate rate. The "granular" fragment is considerably more viscous than the "clear" fragment.

3. The ratio of the amounts of nuclear material and cytoplasm is a determining factor in cleavage rate. Half an egg containing both the egg nucleus and a sperm nucleus cleaves sooner than the normal diploid egg in spite of the injury from cutting. The normal egg cleaves sooner than half an egg containing only the sperm nucleus.

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COLOR INHERITANCE IN LARVÆ OF *CULEX PIPIENS* LINN.

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INTRODUCTION.

In collecting mosquito larvæ from the various types of breeding places, one is often struck by the great variations in color which occur. Many who have collected mosquito larvæ—either Anopheline or Culicine—may recall that there is often a striking similarity between the color of the fully-grown larva and the color of its immediate environment. Larvæ found in water containing large quantities of green organisms will often be brilliant green themselves, while larvæ of the same species if observed in muddy water will be brown. This is not a strict parallelism, however, for one may sometimes find larvæ whose color stands out in contrast to the color of their environment. Two interpretations may be offered of the fact that there is often observed a similarity in the color of the larvæ and the color of their environment. One is the suggestion that the larva ingests the organisms of the water and that the predominating color of the latter manifests itself through the rather transparent integument of the insect. The other is that colors are actually inherited and that the correlation mentioned above is due to the rapid action of natural selection in favor of those larvæ which resemble most closely their environment. Either of these hypotheses is difficult to test unless one employs a species of mosquito which can be bred in captivity from generation to generation. As shown in my previous publications (1927, 1929*a*, 1929*b*) *Culex pipiens* is a very good species for laboratory experimentation. I have now bred this species through 15 generations and in that time have seen it adapt itself to its

[1] National Research Fellow. This research was supported by a grant from the Wellington Fund. I extend thanks here to Dr. L. R. Cleveland for invaluable aid.

laboratory environment in various ways. First, I have shown (1929*b*) that the species must become adapted, through selection, to copulating in captivity, since it copulates in nature while swarming. My strain of mosquitoes will now breed in a space no larger than a test tube, and practically all of the breeding is done in cages constructed from lantern globes. I have also been able to shorten the life-cycle to less than half of what it was when originally brought in from the field. Under optimum conditions of food and temperature larvæ will become fully grown in five days from the time the eggs are laid. While the species thrives better when the females can secure blood meals, I have shown (1929*a*) that the species may be kept breeding on a purely vegetable diet, and recently I have observed one instance of the laying of viable ova by a mosquito in less than 24 hours after her emergence from the pupa and having taken no food of any kind (1929*a*, footnote).

Therefore, when I first observed some vividly green larvæ in my stock I decided to try to determine which of the two hypotheses about larval color would better fit the facts.

Influence of Environmental Factors.

After I had inbred this species for three generations (for purposes shown in another paper (1929*b*)), I noticed the sudden appearance of green larvæ in large numbers. In fact, every larva in one particular breeding bowl was found to be green on the day before pupation. The color persisted in the pupa, although it became obscured as the pigment of the adult tissue developed. Upon emergence the green color could still be observed in the abdomen of the adult. Since I had on hand a bowl of larvæ in which I had seen no trace of green—all of them being reddish brown in color—I decided to save the progenies of the two lots. When ova from each strain were obtained they were allowed to hatch and the larvæ were placed on opposite sides of a bolting cloth partition within a breeding bowl. Stock food consisting of two parts of milk powder to one part of dried blood serum was added in small quantities each day. Thus the larvæ were under practically identical conditions of temperature, food, hydrogen ion concentration, viscosity, etc. In due time the larvæ became fully

grown. All of those on one side of the partition which were from brown stock were brown in color and showed no hint of green coloration. All of those on the other side were vividly green as had been their parents. This seemed to be conclusive proof that environmental factors did not determine the color of the larvæ directly, at least.

Hereditary Behavior.

After the two stocks—brown and green—had been bred three generations and were still breeding true, a male from the brown stock was mated with a female from the green stock. The fully grown larvæ from this cross were "normal" in color; that is, they were predominantly brown with a tint of green visible on the clear parts of the abdomen. The adults from these larvæ were inbred and 10 lots of ova obtained. When they were ready to pupate they were separated into two lots—one containing only those of a pure green color, the other, those showing a pure brown or a color predominantly brown. The results of this cross were as follows:

	Number	Percentage	Ratio
Green	149	25.38	1 : 2.94
Brown	438	74.62	

The brown lot was then subdivided into two groups one of which contained larvæ without any trace of green and the other with traces of green. Due to the rapid pupation of the larvæ it was impossible to determine in this experiment the percentages in the two groups. However, the ones suspected of being heterozygous for these colors were inbred and it was found that the larvæ in this generation were composed of pure green, pure brown, and "normal" individuals. These numbers were too small to be significant quantitatively and are not recorded.

Nature of the Color.

Aside from the experiments described above which show that microorganisms did not determine the color of the larvæ, a number of observations made from larval dissections showed directly that

the color was due to refraction of light from the globules in the fat body. There were no microorganisms observed in the regions showing the colors in question. The fat body, however, is made up of very numerous globules resembling oil which in the aggregate give the colors characteristic of the two strains. The distribution of these colors follows closely the distribution of the fat body within the larva. I am of the opinion that the differences in color of these 4th stage larvæ were due to hereditary differences in the physical or chemical nature of the fat body. One should not confuse the colors about which I have written, with colors in the integument due to pigments.

Conclusions.

On the basis of the results of experiments upon regulation of the environmental factors, upon cross breeding, and upon larval dissections it is concluded that the brown and green colors observed in 4th stage larvæ of *Culex pipiens* are due to hereditary differences rather than to the direct influence of the environment. It is believed that these differences are concerned with the properties of the fat body of the insect—probably with its index of refraction.

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GROWTH REGULATING SUBSTANCES IN ECHINODERM LARVÆ.

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In general we apply the term growth to the progressive development of an organism from the earliest stages to a period approaching maturity. From this series of changes I have selected, as representative, two brief periods namely the early cleavages, and the period of rapid growth in length of the arms of the pluteus during the second and third day of their development. The experiments fall into three groups, the first of which has to do with the direct contact effects of developing eggs upon one another, and the character of the influence of their secretions upon these two periods of growth; the second group deals with the effects of extracts of eggs and of larvæ upon the development of other eggs, and the third is concerned with the possible removal by adsorption and the subsequent recovery of any growth-accelerating and growth-inhibiting substances that may be secreted.

These investigations were begun at Woods Hole in July, 1926, and were continued in the summer of 1927 at Naples where I also undertook a series of experiments with adsorption, the results of which indicate that it is possible by this means to eliminate some of the factors which are involved in the inhibition of developmental processes. It is with pleasure that I acknowledge my indebtedness to Professor Frank R. Lillie who was at that time director of the Marine Biological Laboratory at Wood Hole. Also I am glad of this opportunity to thank the Association for the Promotion of Scientific Research among Women, for the use of the American Woman's Table in the station at Naples. To the director Dr. R. Dohrn I am once more grateful for the many courtesies he has always extended to me while I was a guest of the station.

Materials and Methods.

The forms used at Woods Hole were *Arbacia* and *Asterias*, both being abundant there during the summer months. At Naples the experiments were made on the eggs of *Strongylocentrotus*, *Arbacia*, and *Sphærechinus*. So far as possible eggs and sperms for each series were taken from the same pair of individuals. At Woods Hole the urchins were opened and allowed to shed their eggs and sperm into shallow dishes, with the Mediterranean species the reproductive organs were removed, and the eggs were shaken immediately into fresh sea water. Sperm suspensions were made in the usual manner and the minimal amount used for insemination. At Woods Hole the developing eggs were kept in finger bowls, on a shelf in the laboratory, but in Naples, owing to the great heat it was found that they developed more normally when the dishes which contained them were surrounded with running water. Further methods will be described in connection with individual experiments.

I. The Contact Effect of Developing Eggs and of Larvæ upon the Rate of Cleavage, and upon Growth in Length of Arms of Plutei.

(*a*) *The Influence of Direct Contact.*—It is a matter of ordinary observation that growth of lower organisms, both plant and animal, takes place more rapidly in a crowded medium than it does in a thinly populated culture, provided the food supply is not exhausted. The acceleration in many forms is directly related to the presence of bacteria, and is maintained as long as they are present in large quantities. It was first demonstrated by Wildiers ('01) that isolated yeast cell do not grow and divide, but when a number of cells are added the development proceeds in the normal manner. He pointed out that a secretion of the yeast cells which he termed *bios*, will also produce the same effect. Burrows ('25) and others who have worked extensively with tissue cultures, have observed that isolated cells of the body grow less well *in vitro* than when crowded together. In the fertilized egg of the sea urchin there is sufficient food material to last until the free swimming larvæ develop. There is then, in these cells no need to draw

nutritive materials from the sea water, and food is probably not taken in until the digestive tract is formed. There is strong evidence, however, that the egg secretes substances not necessarily nutritive in nature which greatly modify the composition of the sea water. Whether these substances contain growth hormones, vitamines, or catalysts, it is the purpose of this study to determine.

In order to test the effect of direct contact of one egg upon another the following experiment was made. As soon as the fertilization membrane appeared the inseminated eggs were washed several times in fresh sea water to remove the fertilizin and any sperm remaining in the water. Single eggs were placed in ten to fifty cc. of sea water, in another series five eggs were put in a corresponding volume of water, in another ten eggs, and so on. The rate of cleavage in these eggs was compared with that of several hundred eggs in an equal amount of water. The rate in the isolated eggs was so nearly that of those crowded together that the coefficient of variation in one series was about the same as that of another. All of the eggs reached the free swimming stage at the same time, but the percentage of abnormal eggs was greater when large numbers developed in a proportionally small amount of water. This was no doubt largely due to the excretion of carbon dioxide. Mortality was increased also under these conditions, many of the eggs failing to reach the blastula and gastrula stages.

If, as Robertson ('23) suggests, a catalyst is freed at each nuclear division it would be probable that at the end of the third cleavage only a very minute quantity would have accumulated, but at the end of the 256-cell stage a considerable amount might have passed out into the surrounding medium. In order that such a diffused substance might reach younger developing eggs without direct contact with the older ones, and also to avoid any possibility of confusing the two sets of eggs they were separated by parchment thimbles, collodion membranes, or extremely fine bolting cloth. At first it seemed advisable to bubble air through the water in which the eggs were developing, but this method was abandoned as soon as it was discovered that the eggs in the vessels through which air passed showed a much lower percentage of normal development than those without air. In Table I. the results of a series of these experiments are grouped.

TABLE I.

Type of Membrane Employed.	Stage in Older Eggs.	Min. after Fert. to Cleavage.			Control.		
		I	II	III	I	II	III
Parchment thimble	8-cell	50	70	90	55	80	95
	"	50	75	85	50	80	105
Collodion membrane	Gastrulæ	45	60	90	45	60	90
	"	50	65	85	45	60	90
Bolting Cloth	Plutei	59	94	108	60	95	110
	"	60	95	120	61	93	120

The rate of cleavage in eggs separated from older stages by means of a membrane. The average length of time in minutes was reckoned from insemination until fifty per cent. were divided.

For these experiments the eggs were washed immediately after membrane formation, and placed in the parchment thimble, or in a glass tube 4 cm. in diameter, the lower end of which was covered with the membrane used for the particular series. These dialyzers were then submerged in dishes in which older eggs or embryos had developed up to a given time. As the table shows, there was very little variation in the time of the appearance of the cleavage furrows when the eggs in the dialyzers were compared with the control, but there is some indication of acceleration in the rate of cleavage in the younger eggs, and this acceleration is greater when the membranes were used than when the two sets of eggs were separated by bolting cloth. Whether or not the accelerating substance given off by the older eggs passes through the membrane while the inhibiting substances are held back, remains to be determined when more is known of their nature.

(*b*) *The Effect of Embryo-water upon the Rate of Cleavage and of Growth in Length of Plutei.*—In still another set of experiments eggs were allowed to segment in water in which other eggs had developed up to the gastrula and plutei stages respectively. Springer ('22) used this method in an attempt to determine if so-called formative substances pass from eggs or from larvæ into the surrounding water. She observed no acceleration in rate of cleavage, and her results do not prove or disprove the existence of growth-promoting substances: as she suggests, such substances if present in the egg may not pass into solution in sea

water, or if they do they may be too complex in character to be disassociated from the molecules that hold them. Even if they do pass into the water the cells of the eggs developing in their presence may not be permeable to them. In Fig. 1 the rate of the appearance of the first three furrows is shown in contrast to the rate of cleavage of eggs in embryo-water. The delay here would

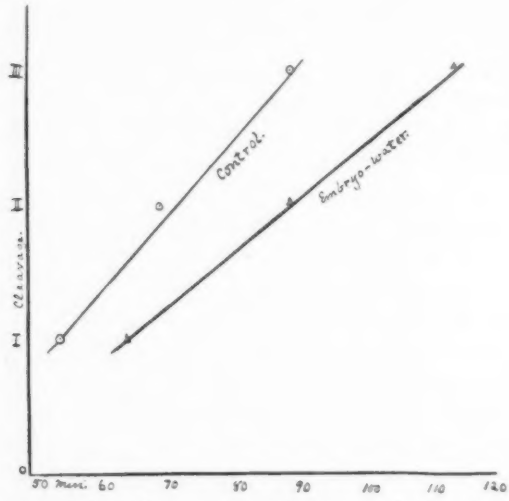

FIG. 1. Comparison of the rate of appearance of the first three cleavage planes (I., II., and III.) in normal eggs (⊙), and in eggs allowed to develop in Embryo-water (△). The abscissa shows the number of minutes from insemination to the appearance of the furrow in 50 per cent. of the eggs. The ordinate gives the cleavages.

indicate that inhibiting substances had accumulated in the water.

Vernon ('95) found that larvæ grown in water in which other larvæ had previously developed were seven per cent. smaller than normal, showing that some of the products of metabolism exert a deleterious effect on growth, but he found that urea and uric acid caused increase in size. He states that a small excess of carbon dioxide stimulated growth, but Haywood ('27) has found that excess of carbon dioxide delays the appearance of the first furrow. In Fig. 2 the average rate of growth in length of the plutei, measuring from the base of the body to the tip of one of the anal

arms is given. The most rapid growth in length takes place during the second and the third days. It will be observed that the greatest delay in growth appears when the eggs develop to the plutei stage in plutei-water, and the delay is less when the older plutei are present, but when younger and older eggs develop together both the young and the old larvæ show retarded growth. Boiling the

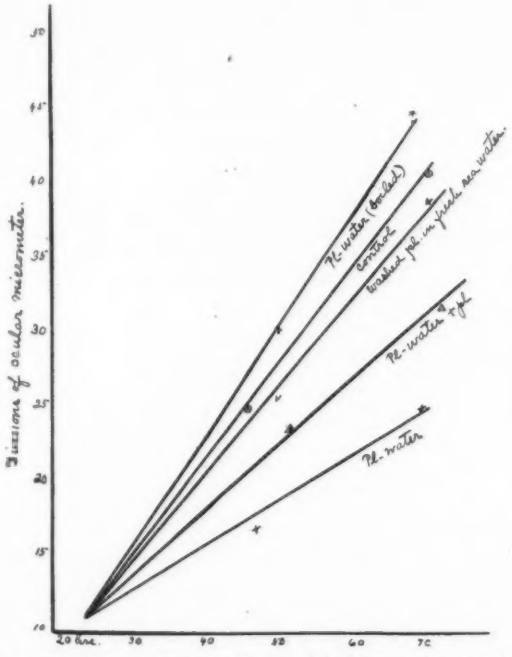

FIG. 2. Comparison of the rate of growth in length of plutei, the abscissa shows the number of hours after insemination, the ordinate, the length of the plutei (body plus anal arm), in divisions of the ocular micrometer.

plutei-water removes the inhibiting effect, and such water standing for a week after the larvæ have been removed seems to have the same effect. We conclude then that embryo-water contains substances that inhibit growth. But some of the inhibiting power is counteracted when living larvæ are present, and if the water in which larvæ have developed is boiled or is allowed to stand long enough, after the larvæ have been removed, the inhibiting effect

is lost. Fig. 2 shows a slight acceleration in the rate of growth in the last two cases. The apparent acceleration in the case of the boiled plutei-water may be due to the adding of distilled water to make up for the loss in boiling.

II. The Effect of Extracts of the Egg and of Larvæ upon the Rate of Cleavage, and upon Later Growth.

As Springer has shown, extracts of eggs and of larvæ made by grinding them with sand and washing out with sea water always

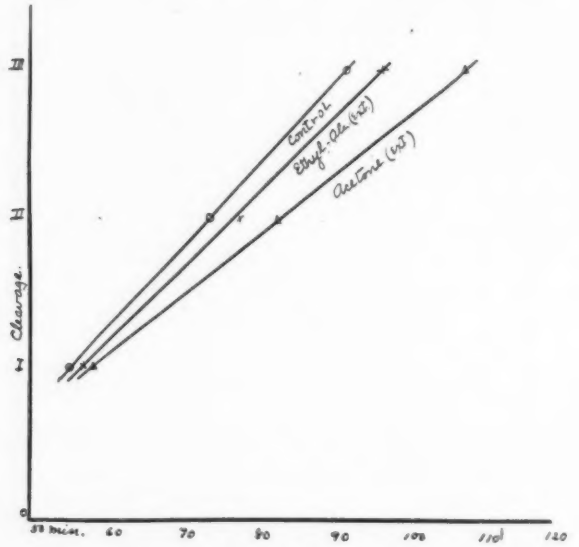

FIG. 3. Time of appearance of the first three furrows in eggs which have developed in alcoholic extract (×), in acetone extract (△), and in sea water (⊙).

inhibit the rate of development. So far I have been unable by dialyzing to remove any substance which stimulates growth, but the results from the following experiments seem to indicate that such substances exist, and they may be masked by the stronger and more abundant growth-inhibiting factors.

In Fig. 3 the cleavage rate is shown for eggs in 90 per cent. ethyl alcoholic and 50 per cent. acetone extract of plutei. It will

be seen at once that the rate is slowed in the alcohol, and slower still in the acetone. In these extract the plutei were placed in the alcohol or acetone, then the mixture without grinding the plutei was evaporated to dryness, and to a given quantity sea water was added. In Fig. 4 the rate of growth in length is given for the second and third day. Here again it is evident that there is

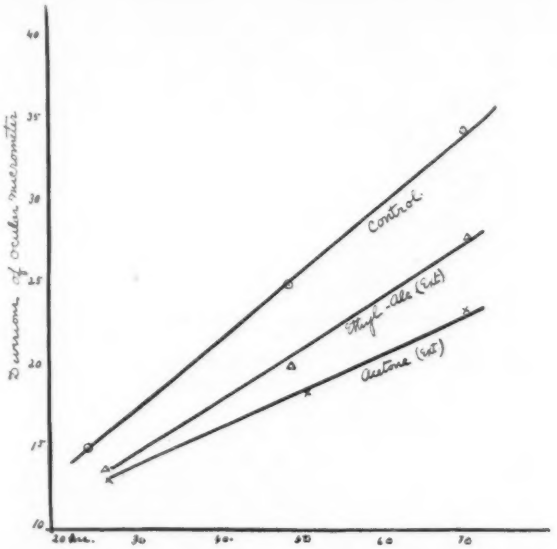

FIG. 4. Growth in length of plutei in the same extracts used in Fig. 3.

greater delay in the acetone extract than there is in the alcohol. If however, the fats are removed from the acetone extract, and a water solution made from the residue there is evidence that the inhibiting effect is removed. This may be seen in Fig. 5.

Heaton ('26) found that yeast and liver cells contain both a substance that inhibits growth and one that promotes it. In extracting these substances he discovered that the factor promoting growth of epithelial cells could be extracted with 97 per cent. alcohol, while the growth-inhibiting substance was soluble in alcohol only up to 75 per cent. I have used these two concentrations of alcohol in making extracts of the plutei of *Strongylocentrotus* and *Arbacia,* but so far I have not found that the extracts

differ in their effect other than that the solutions from the stronger alcohol had a more marked effect than those from the weaker.

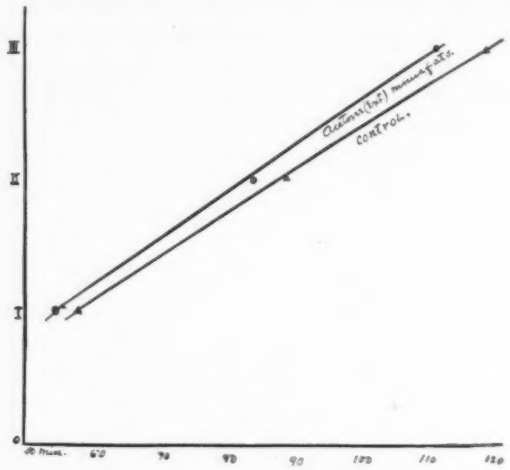

FIG. 5. Cleavage rate in eggs treated with acetone extract from which the fat has been partially removed.

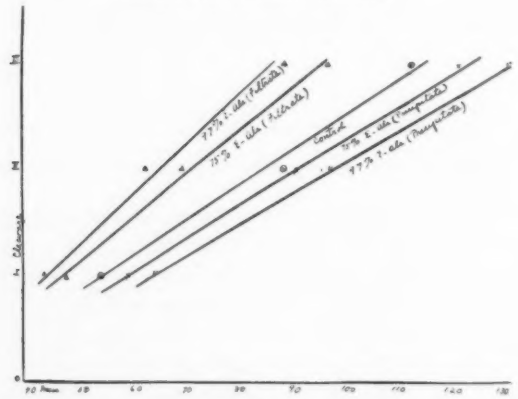

FIG. 6. A comparison of the effect of the filtered alcoholic extracts of plutei (▲ and △) with that of the precipitate (× and ∨).

After allowing the alcohol to evaporate to dryness on the plutei, sea water was added and the precipitate together with the skeletons and the debris removed by filtering. Fig. 6 demonstrates very

clearly that the filtrate and the precipitate differ considerably in their influence on the rate of cleavage, the precipitate delaying it and the filtrate accelerating it.

III. The Removal of Inhibiting Substances by Adsorption.

In order to remove substances secreted by eggs at the time of fertilization, Glaser ('21) used charcoal, and found that the eggs

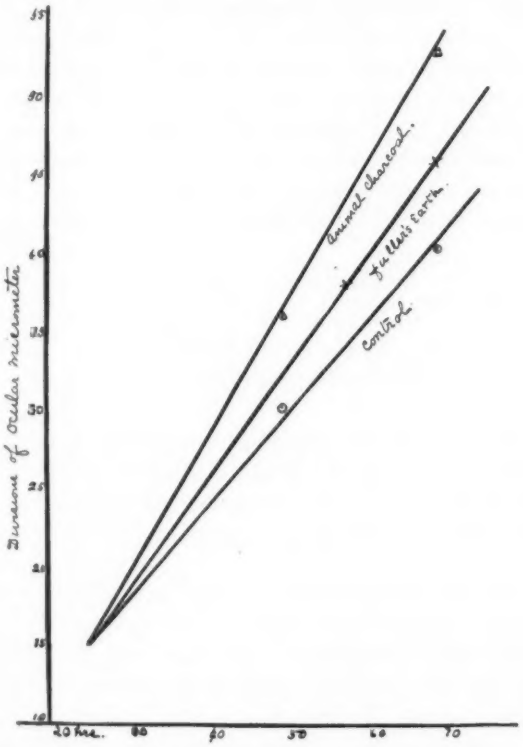

FIG. 7. Growth in length of plutei in the presence of animal charcoal (△), and fuller's earth (×), compared with those growing in sea water (⊙).

were completely sterilized in three to four hours. Hinrichs ('27) also employed charcoal to adsorb fertilizin, which she later recovered with HCl. Baker and Carrel ('27) made use of charcoal,

kaolin, and other adsorbants to remove protein substances from embryo-tissue extract, but they have not yet been successful in recovering the active substances again. In Fig. 7 the rate of growth of plutei is given when animal charcoal and fuller's earth were used as adsorbents. There is evidence here that both of these substances remove some inhibiting influence from the sea water in which the eggs are developing. There is also less abnormality, and the mortality is decreased. The pH of the water was ascertained in these experiments and it varied so slightly from that of the control that it did not seem to be a determining factor. Up to the present time I have not succeeded in removing from the adsorbents any substance which affects the rate of growth of eggs subjected to its influence. It is hoped through longer and more exact investigations to isolate both the growth-inhibiting and the growth-promoting substances in such a form that they may be employed to regulate the development of other eggs.

Summary and Conclusions.

1. The eggs and larvæ of the sea urchin and starfish contain growth regulating substances. These substances pass out into the surrounding water during segmentation, and the later stages of larval development.

2. The nature of these substances has not been determined, but there is some experimental evidence in favor of the conclusion that the inhibiting substances are associated with the lipoid constituents, and the accelerating factor is contained in the protein molecule.

3. After the removal of the fats from the extracts of gastrulæ and plutei, a solution of the residue in sea water exerts a slightly stimulating effect on growth; when the alcoholic extracts are filtered, and the precipitate removed the filtrate has the same accelerating effect. Acetone extracts of eggs and of larvæ, as well as alcoholic extracts, if used in pure form, are inhibitive in their influence.

4. The retarding effect of secretions of growing embryos is removed in the presence of animal charcoal and fuller's earth. The percentage of normal larvæ resulting from eggs grown in the presence of these adsorbents is greatly increased while mortality is decreased.

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STUDIES ON TRANSPLANTATION IN *PLANARIA*.

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During the last two years, the writer has been engaged in a study of transplantation in *Planaria*. The present paper is a brief statement of some of the more important results obtained thus far.

The work was undertaken in the hope of throwing light on various questions. For example: may a grafted piece act as an "organizer" or "reorganizer"? Is its effect species-specific? On what factors does the fate of the graft depend?

MATERIALS AND METHODS.

Planaria dorotocephala Woodworth, and *Planaria maculata* Leidy were the animals used in the work herein reported. The size of the worms used ranges from ten to twenty-two millimeters in length. A weak aqueous solution of chloretone (ca. M/100) was used, in most cases as anaesthetic. The donors were not anaesthetized. In some cases neither the host nor the donor was anaesthetized. In some of the experiments, a triangular hole was made through the body of the host by means of a sharp scalpel at the desired level of the body and a triangular piece of about the same size as the hole from a certain level of the body of the donor was inserted into the hole by means of a capillary pipet, while in other series of experiments a circular hole was made by means of a sharp capillary pipet. The advantage of using triangular pieces and triangular holes in this experiment is very obvious, in as much as the polarity of the graft and of the host was one of the points under investigation. The operated worms were kept in the dark in moist chambers consisting of fingerbowls and small watch glasses with very little well-water, only enough to keep the worms moist, for about twelve to twenty-four hours immediately following the operation. After this length of time,

those with successful grafts were examined under a binocular microscope; counted, recorded and transferred into fingerbowls with ca. 200 cc. fresh well-water, covered with glass plates and kept in the dark in the usual way. In some of the experiments, the host was beheaded to keep it relatively quiet and thus increase the per cent. of "takes," while in other cases, the host was not beheaded. Both homoioplastic transplantation and heteroplastic transplantation were performed the latter between *Planaria dorotocephala* and *Planaria maculata*. In some of the experiments *P. maculata* was the host, while in others the host was *Planaria dorotocephala*. Head, prepharyngeal, pharyngeal, postpharyngeal, and tail regions were the different levels of the body that have been used both as graft and as region of insertion in these experiments. The grafted piece was either transplanted without its axis being rotated, or the axis of the transplanted piece was rotated 60–180 degrees, and in some cases, the piece was grafted with its dorsal surface toward the ventral surface of the host. Histological studies of the results are being made and will be reported elsewhere.

Experiments and Results.

Portions of the head (Figs. 1, 2, 3) of *Planaria dorotocephala* similar to those used as head grafts were isolated. Result: As a rule, these isolated portions of the head, if not too small, remained alive for several days or longer but did not reconstitute postcephalic region (Figs. 4, 5).

When a piece of the ganglionic region of the head above certain size (Figs. 1, 2, 3) is transplanted into the prepharyngeal region very near the head of the host, it reconstitutes a head of some sort which may later be resorbed, or may become detached, or in case the host is beheaded for the second time, it may develop as in Figs. 6, 7, and 8, with very little or no outgrowth from the host tissue. And if the host is beheaded for the second or third time after transplantation, the grafted head usually decreases the head frequency of the host. If the cut in the second or third beheading is very close to the region of the graft, head formation at the cut surface may be completely inhibited; and in this case, the grafted head acts as the head of the host (Fig. 9). If the inhibition of head formation at the cut surface at the third beheading

is not complete, the two heads may approach each other and sooner or later fuse together (Fig. 10).

A piece of the ganglionic region of the head (Figs. 1, 2, 3), if not too small, when transplanted into the prepharyngeal region, is not resorbed, but is capable of reconstituting a complete head and of inducing the development of an outgrowth (Figs. 11–15), and thus a new axis is formed with the grafted piece at its tip.

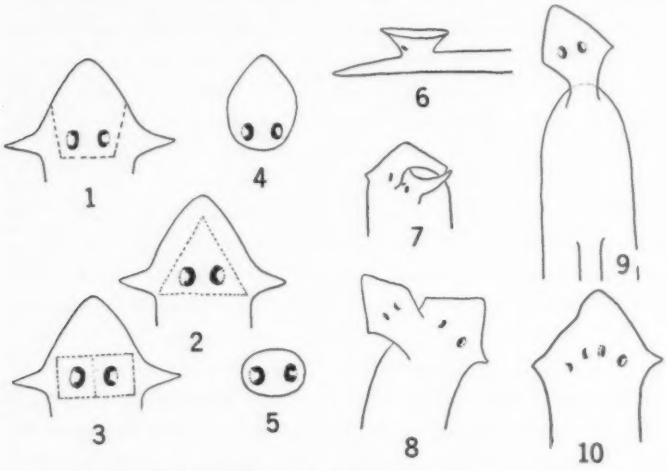


FIG. 1. Head of *Planaria* showing the part transplanted.
FIG. 2. Head of *Planaria* showing the part transplanted.
FIG. 3. Head of *Planaria* showing the part transplanted.
FIG. 4. Isolated portion of the head of *Planaria dorotocephala*, 12 days after isolation.
FIG. 5. Isolated portion of the head of *Planaria dorotocephala*, 12 days after isolation.
FIG. 6. Side view of *Planaria dorotocephala* with headgraft.
FIG. 7. *Planaria dorotocephala* with head-graft.
FIG. 8. *Planaria dorotocephala* with head-graft.
FIG. 9. *Planaria dorotocephala* with head-graft from *P. maculata*.
FIG. 10. *Planaria dorotocephala* with grafted head from *P. dorotocephala* fused with the regenerated head of the host, after the third beheading.

A piece of the ganglionic region of the head transplanted to a region very near the head of the host does not act as an organizer. When transplanted to more posterior regions, if not resorbed, it acts as an organizer. When transplanted into the postpharyngeal region near the tail, if not resorbed, it is capable of inducing the

formation of a secondary pharynx in the posterior region, and of reversing the polarity of the region originally anterior to the level of the graft. This reversal of polarity is shown by the development of a new pharynx opposite in direction to the original pharynx and by complete reorganization of the alimentary tract. It may also inhibit head formation at the cut surface several millimeters

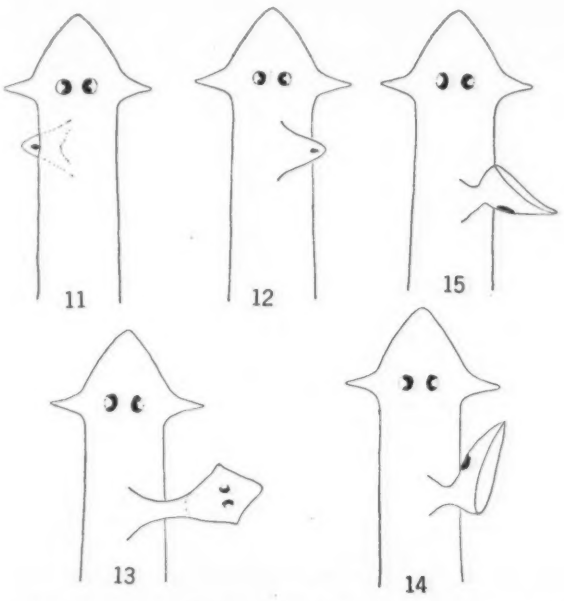


FIG. 11. *P. dorotocephala* with head-graft developing on the ventral surface of the host. Graft is partly inhibited.

FIG. 12. *P. dorotocephala* with head-graft developing on the dorsal surface of the host. Graft is partly inhibited.

FIG. 13. *P. maculata* with head-graft from *P. dorotocephala.*

FIG. 14. *P. maculata* with head-graft from *P. dorotocephala.*

FIG. 15. *P. maculata* with head-graft from *P. dorotocephala.*

anterior to the graft after the anterior part of the host is removed for the second, third or fourth time, posterior or through the pharynx. The result in these cases apparently depends upon the physiological activity of the level of the body of the host and of

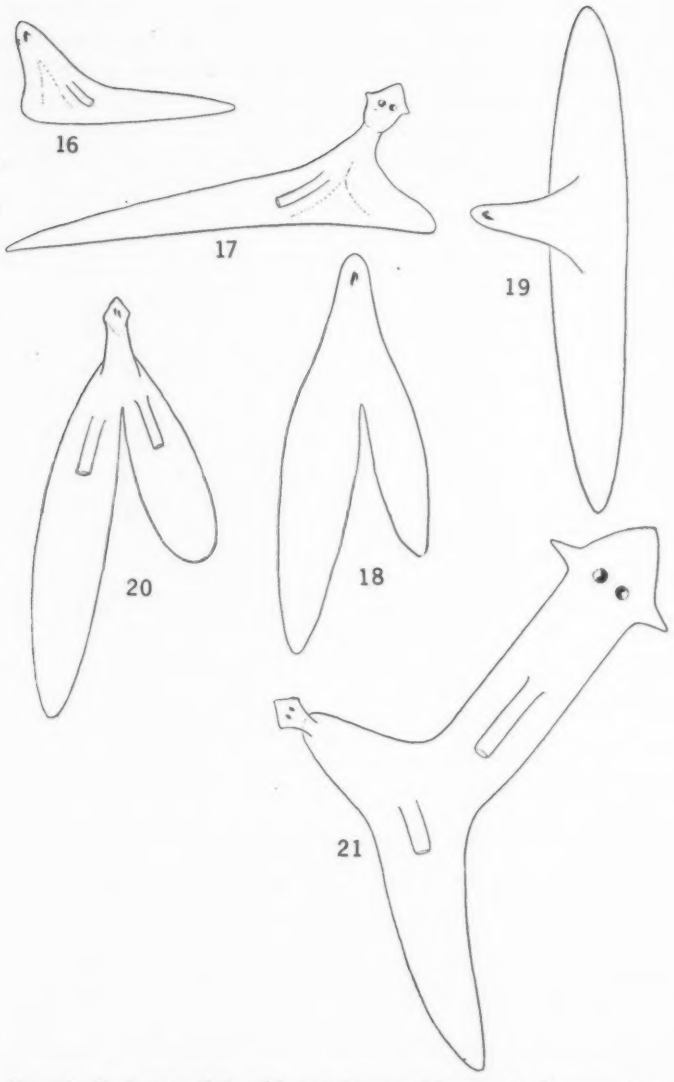
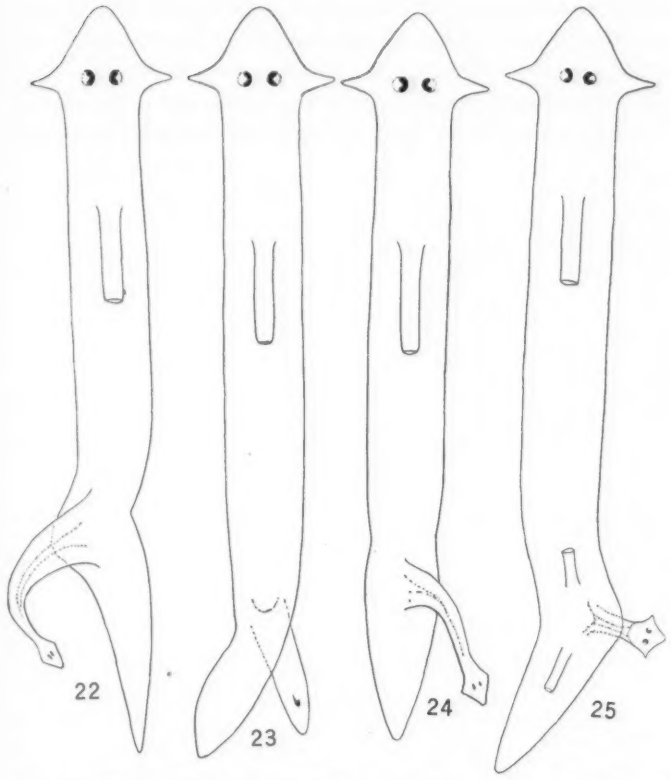


FIG. 16. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala*. Side view showing the induced pharynx.

FIG. 17. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala*. Side view showing the induced pharynx.

FIG. 18. *P. dorotocephela* with postpharyngeal head-graft from P. *doro-*



tocephala. Regeneration of head at the anterior cut surface is prevented, and showing reversal of polarity.

FIG. 19. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala*. Regeneration of head at the anterior cut surface is prevented, and showing reversal of polarity.

FIG. 20. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala*. Regeneration of head at the anterior cut surface is prevented, and showing induced secondary pharynges and reversal of polarity.

FIG. 21. *P. maculata* with postpharyngeal head-graft from *P. dorotocephala*. Showing the induced secondary pharynx.

FIG. 22. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala*, showing the induced outgrowth from host tissue.

FIG. 23. *P. dorotocephala* with postpharyngeal head-graft from *P. maculata*.

FIG. 24. *P. dorotocephala* with postpharyngeal head-graft from *P. maculata*.

FIG. 25. *P. maculata* with postpharyngeal head-graft from *P. maculata*, showing the two induced pharynges and reversal of polarity.

the grafted head and of the species used as host. Cross transplantation between *Planaria maculata* and *P. dorotocephala* shows that this capacity to reverse the polarity and to induce the development of secondary pharynges in the posterior region of the host is not species-specific, for the head of *P. dorotocephala* is capable of

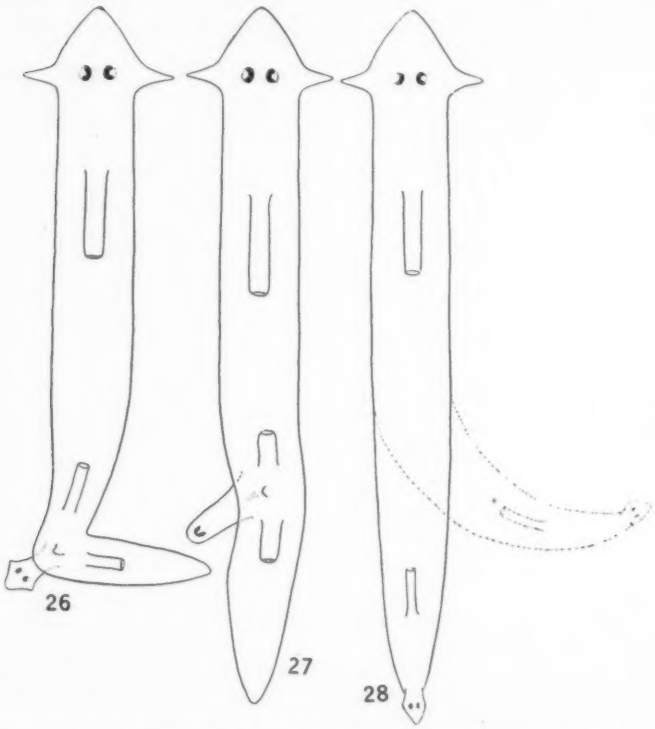


FIG. 26. *P. dorotocephala* with postpharyngeal head-graft from *P. maculata*, showing the induced pharynges and reversal of polarity.
FIG. 27. *P. maculata* with head-graft from *P. maculata*, showing the induced secondary pharynges and reversal of polarity.
FIG. 28. *P. dorotocephala* with head-graft from *P. maculata*, showing the induced pharynx and reversal of polarity.

inhibiting head formation at the cut surface several millimeters anterior to it, reversing the polarity of the region anterior to it, and inducing the formation of secondary pharynges (Figs. 16–31)

in *P. maculata* as host and vice versa. Feeding experiments show that these secondary pharynges induced by the grafted head, are functional, *i.e.*, they are extruded and attached to the piece of liver and show the normal peristalsis of the pharynx characteristic of the feeding reaction of *Planaria*.

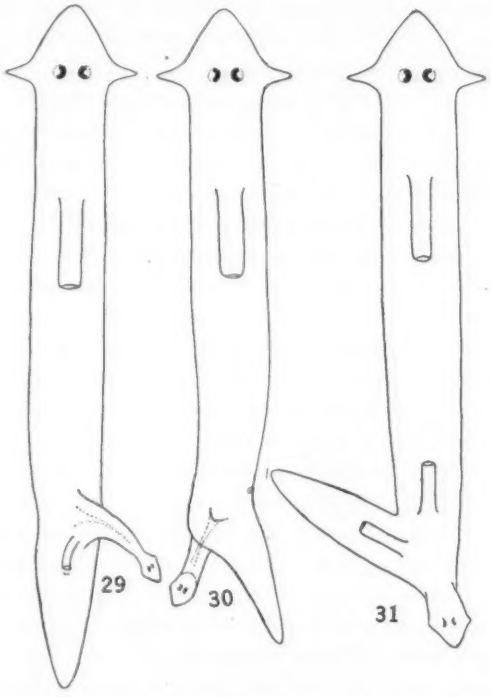


FIG. 29. *P. dorotocephala* with head-graft from *P. maculata*, showing the induced secondary pharynx and outgrowth from host tissue.

FIG. 30. *P. dorotocephala* with head-graft from *P. dorotocephala*, showing outgrowth from host tissue.

FIG. 31. *P. dorotocephala* with head-graft from *P. dorotocephala*, showing induced secondary pharynges and reversal of polarity.

Discussion.

Considering the results of all the experiments performed not merely of those herein reported, the writer is of the opinion that the fate of the graft does not depend primarily upon its degree of specialization, but rather upon the degree of physiological activity of the graft, and of the region of the host into which the piece is implanted, *i.e.*, if the degree of physiological activity of the graft is very high with respect to the region of the host where it is grafted, the graft may not be resorbed, particularly when it is far from the dominant region of the host. For example, when a portion of the head (an example of a piece of the body with a very high degree of physiological activity) is transplanted into a region very near the head of the host, the activity of the grafted head may be decreased by the dominant head of the host and it may finally be resorbed, or else fusion of the two heads may take place.

In *Planaria*, the head is the most active and hence the most dominant region of the body. This fact has been experimentally demonstrated, qualitatively and quantitatively, by Child and confirmed by others in different species of animals besides *Planaria dorotocephala*. Rand and Ellis (1926) working on *P. maculata*, had confirmed the dominance of the head. They termed it "inhibitory dominance." Rand and Browne (1926) working on *P. maculata* had demonstrated that the presence of a grafted head may inhibit the regeneration of a head at an exposed anterior cut surface. The results of some of the experiments herein reported confirm this finding of Rand and Browne.

The head of some sort developed from a graft consisting of a portion of the ganglionic region may not only inhibit the development of a head at a cut surface some distance anterior to it, but may completely reverse the polarity of a region anterior to its level and may induce the reorganization of a region posterior to its level with the formation of a secondary pharynx. Evidently the graft from the ganglionic region is able to act as an "organizer" or more strictly speaking, as a "reorganizer" (Child, 1929) of other regions of the body into which it is implanted.

Grafts of other regions of the body may in some cases give rise to outgrowths. Whether or not such outgrowth develop in particular cases depends on various factors, *e.g.*, size and orientation of graft, level from which it is taken and region into which it is implanted. Further investigation is necessary, however, to determine to what extent a real reorganization occurs in such outgrowths.

SUMMARY.

1. A new technique of transplantation in *Planaria* which gives a high per cent. of "takes" is described.

2. The head, or part of it, which is the most active region of the body of *Planaria,* when trannsplanted into the postpharyngeal region or relatively less active region of the body, is capable, not only of acting as an organizer, and completely reversing the polarity of the region anterior to it, but also inhibiting the formation of a head at a cut surface several millimeters anterior to it.

3. Both homoioplastic and heteroplastic transplantations were performed in *Planaria dorotocephala* and *P. maculata.* The results of the experiments on heteroplastic transplantation between the two species used, show that the action of the graft is not species-specific.

4. The results of the experiments indicate that the fate of the graft does not depend primarily upon the degree of its specialization but on the degree of physiological activity of the level of the body from which it is taken and also on the region of the body of the host in which it is implanted.

5. The fate of the graft also depends upon the size of the graft, degree of "take," and orientation of the graft in the body of the host.

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